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INSTRUCTIONS TO AUTHORS

The Biological Bulletin accepts outstanding original research reports of general interest to biologists throughout the world. Papers are usually of intermediate length (10–40 manuscript pages). Very short papers (less than 10 manuscript pages including tables, figures, and bibliography) will be published in a separate section entitled "Short Reports." A limited number of solicited review papers may be accepted after formal review. A paper will usually appear within four months after its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review.

1. ***Manuscripts.*** Manuscripts, including figures, should be submitted in triplicate. (Xerox copies of photographs are not acceptable for review purposes.) The original manuscript must be typed in double spacing (*including* figure legends, footnotes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. Manuscripts should be proofread carefully and errors corrected legibly in black ink. Pages should be numbered consecutively. Margins on all sides should be at least 1 inch (2.5 cm). Manuscripts should conform to the *Council of Biology Editors Style Manual*, 4th Edition (Council of Biology Editors, 1978) and to American spelling. Unusual abbreviations should be kept to a minimum and should be spelled out on first reference as well as defined in a footnote on the title page. Manuscripts should be divided into the following components: Title page, Abstract (of no more than 200 words), Introduction, Materials and Methods, Results, Discussion, Acknowledgments, Literature Cited, Tables, and Figure Legends. In addition, authors should supply a list of words and phrases under which the article should be indexed.

2. ***Figures.*** The dimensions of the printed page, 7 by 9 inches, should be kept in mind in preparing figures for publication. We recommend that figures be about 1½ times the linear dimensions of the final printing desired, and that the ratio of the largest to the smallest letter or number and of the thickest to the thinnest line not exceed 1:1.5. Explanatory matter generally should be included in legends, although axes should always be identified on the illustration itself. Figures should be prepared for reproduction as either line cuts or halftones. Figures to be reproduced as line cuts should be unmounted glossy photographic reproductions or drawn in black ink on white paper, good-quality tracing cloth or plastic, or blue-lined coordinate paper. Those to be reproduced as halftones should be mounted on board, with both designating numbers or letters and scale bars affixed directly to the figures. All figures should be numbered in consecutive order, with no distinction between text and plate figures. The author's name and an arrow indicating orientation should appear on the reverse side of all figures.

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4. ***A condensed title*** or running head of no more than 35 letters and spaces should appear at the top of the title page.

5. ***Literature cited.*** In the text, literature should be cited by the Harvard system, with papers by more than two authors cited as Jones *et al.*, 1980. Personal communications and material in preparation or in press should be cited in the text only, with author's initials and institutions, unless the material has been formally accepted and a volume number can be supplied. The list of references following the text should be headed LITERATURE CITED, and must be typed double spaced on separate pages, conforming in punctuation and arrangement to the style of recent issues of *The Biological Bulletin.* Citations should include complete titles and inclusive pagination. Journal abbreviations should normally follow those of the U. S. A.

Standards Institute (USASI), as adopted by BIOLOGICAL ABSTRACTS and CHEMICAL ABSTRACTS, with the minor differences set out below. The most generally useful list of biological journal titles is that published each year by BIOLOGICAL ABSTRACTS (BIOSIS List of Serials; the most recent issue). Foreign authors, and others who are accustomed to using THE WORLD LIST OF SCIENTIFIC PERIODICALS, may find a booklet published by the Biological Council of the U.K. (obtainable from the Institute of Biology, 41 Queen's Gate, London, S.W.7, England, U.K.) useful, since it sets out the WORLD LIST abbreviations for most biological journals with notes of the USASI abbreviations where these differ. CHEMICAL ABSTRACTS publishes quarterly supplements of additional abbreviations. The following points of reference style for THE BIOLOGICAL BULLETIN differ from USASI (or modified WORLD LIST) usage:

A. Journal abbreviations, and book titles, all underlined (for *italics*)

B. All components of abbreviations with initial capitals (not as European usage in WORLD LIST *e.g. J. Cell. Comp. Physiol.* NOT *J. cell. comp. Physiol.*)

C. *All* abbreviated components must be followed by a period, whole word components *must not* (*i.e. J. Cancer Res.*)

D. Space between all components (*e.g. J. Cell. Comp. Physiol.*, not *J.Cell.Comp.Physiol.*)

E. Unusual words in journal titles should be spelled out in full, rather than employing new abbreviations invented by the author. For example, use *Rit Vísindafjélags Islendinga* without abbreviation.

F. All single word journal titles in full (*e.g. Veliger, Ecology, Brain*).

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H. A few well-known international journals in their preferred forms rather than WORLD LIST or USASI usage (*e.g. Nature, Science, Evolution* NOT *Nature, Lond., Science, N.Y.; Evolution, Lancaster, Pa.*)

6. ***Reprints, charges.*** Authors will be charged the excess over $100 of the total of (a) $30 for each printed page beyond 15, (b) $30 for each table, (c) $15 for each formula more complex than a single line with simple subscripts or superscripts, and (d) $15 for each figure, with figures on a single plate all considered one figure and parts of a single figure on separate sheets considered separate figures. Reprints may be ordered at time of publication and normally will be delivered about two to three months after the issue date. Authors (or delegates for foreign authors) will receive page proofs of articles shortly before publication. They will be charged the current cost of printers' time for corrections to these (other than corrections of printers' or editors' errors).

Editor's Note

This year, 1988, marks the Centennial of the Marine Biological Laboratory. In celebration of this anniversary, *The Biological Bulletin* has reprinted—with the permission of the University of Chicago Press—F. R. Lillie's (1944) book, *The Woods Hole Marine Biological Laboratory* as a supplement to this (February, 1988) issue. Not only is 1988 a year to celebrate the laboratory's accomplishments, but it is an appropriate time to recognize its journal, *The Biological Bulletin.* It is also an opportune time to make any changes in *The Biological Bulletin* that seem appropriate to the times and to reaffirm its editorial policy. Accordingly, the cover of *The Biological Bulletin* has been redesigned in keeping with modern trends and the page size has been increased from 5 inches to 7 inches. This permits two column printing for increased ease of reading.

These changes in format may seem drastic to some. However, no significant changes in editorial policy are planned. *The Biological Bulletin* will remain a journal of general biology. Manuscripts from all fields of biology, be they animal or plant, aquatic or terrestrial, descriptive or experimental are considered for publication. This, then, will continue the editorial policy that has obtained throughout *The Biological Bulletin's* history. This very interesting history of *The Biological Bulletin* is reviewed by Ms. Clapp, Assistant Editor.

—Charles B. Metz, Editor

Reference: *Biol. Bull.* **174:** 1–3. (February, 1988)

The History of *The Biological Bulletin**

PAMELA L. CLAPP

Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Just nine years younger than the Marine Biological Laboratory (MBL) itself is *The Biological Bulletin*—the MBL's own publication and one of America's first scientific journals. Physically, little has changed since its inception as the *Zoological Bulletin* in the summer of 1897, in spite of an evolution of scientific thought and method over the past 91 years. Many of these developments have been chronicled between the familiar covers of *The Biological Bulletin.*

The Early Years

As first Director of the MBL and editor of the specialized *Journal of Morphology,* C. O. Whitman recognized the need for a more general zoological journal—specifically, one published in the United States which might eventually compete with the prestigious European journals of the day. He felt that an outlet was needed for brief, primarily descriptive scientific papers—one that would attract authors by offering them rapid publication of their work.

Word spread quickly about the new journal, and within a year the first issue of the *Zoological Bulletin* had been published with Whitman and Prof. W. M. Wheeler as co-editors. Five more numbers were published, and by the late spring of 1898 Volume 1 was complete. A second volume was published the following year. The papers included in these volumes were relatively short, descriptive works. Some very brief methods papers, consisting of a paragraph or two, were also included.

Although Whitman was the director of the MBL at the time, no official relationship existed between the MBL and the *Zoological Bulletin.* Apparently Whitman and others felt that both the journal and the MBL would benefit by a closer association between the two. In 1899 a new publication appeared in place of the *Zoological Bulletin.* The new title—*The Biological Bulletin*—placed the journal in an even more general market, and the statement, "Edited by the director and members of the staff of the Marine Biological Laboratory," clearly indicated the new journal's tie to the MBL.

Unfortunately, problems arose after the appearance of the first two volumes of *The Biological Bulletin.* Negotiations with Ginn & Company, the printer Whitman used for both the *Bulletin* and the *Journal of Morphology,* ended with Whitman's decision to contract with another printing company. Time was needed to regroup and reorganize the journal.

After almost two years of inactivity, publication of the *Bulletin* resumed in June, 1902, with the New Era Publishing Company of Lancaster, Pennsylvania (now Lancaster Press) as the printer. The "new" *Bulletin* also had an appointed editorial board and a managing editor. The staff members comprised a very distinguished group: C. O. Whitman, E. G. Conklin, Jacques Loeb, T. H. Morgan, W. M. Wheeler, and E. B. Wilson—all notable scientists and founding fathers of the MBL. Heading the group as Managing Editor was Frank R. Lillie, whose term as editor lasted 25 years.

Upon resumption of publication, the *Bulletin*'s editors made the following statement in a prospectus published in 1902:

> "There is in America no journal that takes the place of the *Biologisches Centralblatt* or the *Anatomischer Anzeiger* in Germany, although there is an abundance of material to support such a publication. It is hoped that the *Bulletin* may occupy this field, and meet the need for rapid publication of results . . . the *Bulletin* will undoubtedly meet a real need; but the responsibility for its success rests with American biologists, and the editors, therefore confidently appeal to them for their support."

Eighty-six years later, it is apparent that the editors' appeal met with success. With the exception of minor printing delays—primarily occurring during World War II—two volumes of *The Biological Bulletin* have been published yearly, with numbers appearing monthly to 1929 and bi-monthly thereafter. Through the years the tradition of quality publication has continued regardless of outside social or economic adversities. Each new editor—of which there have been six since Lillie—has brought his own expertise and dedication to the journal.

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* This article originally appeared in Vol. 3, Nos. 1 and 2 of the MBL newsletter, *The Collecting Net.*

Following Carl R. Moore's brief term (1927–1930), Alfred C. Redfield became editor of the *Bulletin* in 1930. During his tenure, Redfield skillfully guided the *Bulletin* through the depression years. The country's economic climate had little effect on the journal, which remained financially sound and sometimes even showed a small profit. The number and quality of submitted manuscripts varied only slightly during that period. Redfield attributed much of the journal's success to the members of the MBL Corporation, who contributed forty percent of the original research articles published between 1930 and 1940.

The War Years

H. B. Steinbach succeeded Alfred C. Redfield as editor of *The Biological Bulletin* in 1942, just in time to experience some of the more trying years in the journal's history. Because of the war, the government relied on Lancaster Press to juggle increased printings with their civilian accounts. As a result, *Bulletin* issues were often delayed and reprints were rarely printed promptly. Paper supplies for non-government purposes were very low, prompting the *Bulletin* to reduce type size and page margins. These changes, coupled with a significant decrease in submissions, resulted in noticeably smaller volumes throughout the war years.

The war also caused severe problems with the distribution of publications with foreign mailings. Prior to America's direct involvement in the war, dangerous shipping conditions forced Lancaster Press to hold back all foreign subscriptions. Once the U. S. had entered the conflict, mailing resumed, but was seriously restricted—licenses were required for all foreign mail. And of course, no mail could be sent to "enemy, enemy controlled, or enemy occupied" areas.

Peace brought additional problems—or compounded old, primarily financial ones—and Steinbach continued his own good-natured "battle" with Lancaster Press. Post-war printing costs skyrocketed, and with every new year came notice of Lancaster's impending price increases. On December 15, 1947 Steinbach wrote to the president of Lancaster Press:

> "I was very interested to receive your latest literary effort (December 2) and will bind it in my files as another elegant example of how one may entreat a horrible subject in a relatively painless fashion. As I have remarked before, your letters, each of which costs the MBL some hundreds of dollars, are masterpieces of exposition with just the right touch of sympathy that almost makes me feel that it is an honor to be charged more by the Lancaster Press."

Despite his efforts, the price increases (as always seems to be the case) were unavoidable.

When Donald P. Costello assumed the editorship in 1951, the *Bulletin* was well on its way to recovering from the strains of war. Every year saw the submission of more manuscripts, and by 1955 Costello was receiving almost twice as many papers as were submitted in the mid-1940's. To prevent a backlog of manuscripts—something that would have naturally slowed publication—Costello became increasingly selective. In his 1965 annual report to the Corporation, he noted a 59 percent acceptance rate for the year as compared with 61 percent and 84 percent in 1955 and 1945, respectively. The *Bulletin*'s reputation for scientific excellence, rapid publication, and inexpensive page charge policies made the journal quite appealing to many authors.

As authors increased, subscriptions increased as well. The *Bulletin*'s press run more than doubled during Costello's editorship, from 1300 when he began to 3000 upon his retirement in 1969. The *Bulletin* gradually assumed "a more international role in biological publications, both in the widening of its subscription list and in the accepted contributions" of foreign readers and authors.

The Era of the Specialty Journal

By the time W. D. Russell-Hunter took the helm of the *Bulletin* in 1969, the era of the specialty journal had arrived. These journals offered a quick reading on a limited subject to a select audience. Many investigators, convinced that publication in these specialty journals was the way of the future, undoubtedly prodded Russell-Hunter to consider making the *Bulletin* more specialized during this period. Russell-Hunter believed, however, that:

> "if one regards [*The Biological Bulletin*] as a biological organism, it would seem good evolutionary strategy to maintain and encourage diversity. Long-term fitness, given the selection pressures which can bring about the decline of a specialist journal within a decade of its founding, to ensure the *Bulletin*'s surviving for another 140 volumes may be conferred by a moderately eclectic editorial policy."

With more experimental disciplines turning to the specialty journal, the *Bulletin* had to be more competitive in certain areas to attract a diverse following. For instance, electron microscopy had become a major research tool of biology, increasing the importance of quality photographic reproduction. Russell-Hunter quickly moved to improve the *Bulletin*'s photo reproduction by upgrading paper stocks, photographic screens, and platemaking techniques used in the printing process.

The final year of Russell-Hunter's distinguished tenure, 1979, coincided with the revolutionary effects of the computer age on the printing industry: namely the introduction of photo-offset printing and computerized typesetting. The *Bulletin* had always been printed by the less efficient and economical letterpress method, but with Russell-Hunter's usual effectiveness the change proved to be a smooth one. The *Bulletin* was aging gracefully.

Recent History

Charles B. Metz had enjoyed a long and memorable association with *The Biological Bulletin* even before he became editor of the journal in 1980. His first published research paper appeared in the June 1942 issue, and he remained a regular contributor to the journal until his retirement from scientific research. As did his predecessors, Metz first became officially involved with the *Bulletin* as an editorial board member.

Upon his appointment as editor, Metz immediately initiated the *Bulletin*'s invited review article program. Publication of "state-of-the-art" papers, each reviewing a different aspect of biology, was designed to heighten subscribers' awareness of the breadth of articles included in *The Biological Bulletin*. More importantly, Metz hoped the program would attract an even greater number of top-notch papers—especially those by members of the MBL and Woods Hole scientific community. To date, 22 such reviews dealing with neurobiology, physiology, ecology, and developmental biology—to name a few—have been published. A quick glance at the table of contents of a recent issue reveals that the review article program has helped to attract quality articles on a variety of subjects.

Of course there is much work left to be done, even as the *Bulletin* inches its way up to the #1 spot on Jane Fessenden's MBL library readership survey (it's currently #10 out of 4700 journals!). Finding willing review writers can be difficult—the most desirable authors are always the most professionally over-committed. Convincing potential authors that, yes, the *Bulletin does* publish articles in their area of interest and that their papers *will* be received by a broad audience, is also an on-going effort.

But the effort appears to be paying off. Today one-third of the copies of each issue are mailed abroad, and over the past year foreign authors accounted for approximately twelve percent of the published papers. The MBL library sends out 400 *Bulletin* subscriptions each year to organizations and other publications for which they receive approximately 600 subscriptions to various journals and publications in return. With the press run averaging about 2500, the *Bulletin* can be found in most major colleges and universities in the U. S. and abroad. And of course the *Bulletin* is also received by all MBL Corporation members.

In summary, C. O. Whitman's dream has become reality—the MBL's "little journal" is truly an international success.

Reference: *Biol. Bull.* **174:** 4–19. (February, 1988)

Larval Form and Metamorphosis of a "Primitive" Sea Urchin, *Eucidaris thouarsi* (Echinodermata: Echinoidea: Cidaroida), with Implications for Developmental and Phylogenetic Studies

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Abstract. The order Cidaroida (Echinodermata, Echinoidea) is universally recognized as an ancient (~230 mya) lineage and is thought to be the sister group to the more modern euechinoids. The present study on *Eucidaris thouarsi* corroborates earlier findings that cidaroids have a characteristic larval form that is different from that of euechinoids and gives the first detailed description of juvenile rudiment formation and metamorphosis in a cidaroid. Larvae of *E. thouarsi* lack an amniotic invagination (vestibule), have many (~20) juvenile spines on the larval epidermis and do not histolyze the entire larval epidermis at metamorphosis. Consequently, metamorphosis of cidaroid larvae is simple when compared to that of euechinoids. In larvae of *E. thouarsi,* epithelial cells appear to grow over the epidermis that becomes radial nerve tissue, but this process is not visible externally and may occur by a different mechanism than that reported for euechinoids. Typical development and metamorphosis of the class Echinoidea is usually represented by the euechinoids of the family Echinidae. The present study shows that feeding larvae of echinoids have greater variability than previously recognized in developmental patterns and processes, including differences in the fates of larval epidermal tissues and the timing of production of adult spines. The growth of podia exposed on the left side of the larval body is strikingly similar between cidaroid and asteroid larvae and is an example of probable convergence of characters among the echinoderms. The absence of a vestibule in cidaroids also raises uncertainties about the homology of this structure across the phylum Echinodermata.

Introduction

Living echinoids form two distinct lineages, the Cidaroida and the Euechinoidea (Jensen, 1981; Smith, 1984a). Members of the extant family Cidaridae have a fossil record extending back into the Triassic (Karnian, *ca.* 230 million years ago). The cidarids are thought to have evolved from the miocidarids which had flexible tests with biserially arranged plates in each ambulacrum and interambulacrum, and interambulacral lantern supports called apophyses (Durham, 1966; Kier, 1977a; Jensen, 1981). Because miocidarids are the only echinoids known to have crossed the Permo-Triassic boundary, miocidarids are the presumed ancestors of the Euechinoidea (Durham, 1966; Kier, 1977a; Smith, 1984a). Recently, Kier (1984) showed that many of the Triassic echinoids lacked apophyses and therefore were not cidarids or miocidarids. Because some of these non-cidaroid Triassic echinoids showed slight development of ambulacral lantern supports (auricles), Kier (1984) proposed that the cidaroids and euechinoids actually separated prior to the first occurrence of the miocidarids, known from the Permian of Europe and North America (Kier, 1965, 1974).

Even though cidaroids and euechinoids have long been separate lineages and extant cidaroids are diverse (>140 species, Kier, 1977b), only a few studies have examined cidaroid larval development (*e.g.,* Prouho, 1887; Mortensen, 1921, 1937, 1938; Tennent, 1914, 1922; Schroeder, 1981). Mortensen (1938) reared *Prionocidaris baculosa* through metamorphosis; he described the

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larva and the juvenile, but gave no detailed descriptions of juvenile rudiment formation or metamorphosis. McPherson (1968) described the larval development of *Eucidaris tribuloides* and included photographs of a larva and a juvenile (Figs. 23 and 24); however, the specimens in these photos are probably not cidaroids (see Discussion). Mortensen (1927) also described post-larval development in several cidaroids by examining small juveniles collected from the field. Barker (1985) provided a short description of the development of brooded embryos of *Goniocidaris umbraculum*. In contrast, over 45 species of euechinoids have been reared through metamorphosis (see Emlet *et al.*, 1987; Chia and Burke, 1978), and these studies provide the basis for the classical descriptions of echinoid development and metamorphosis (MacBride, 1903, 1918; von Ubisch, 1913; Hyman, 1955; Okazaki, 1975).

Recently, Schroeder (1981) studied the development of *Eucidaris tribuloides* from fertilization to the two-armed larval stage. He identified several unique aspects of development, confirmed Tennent's (1914) earlier observations that this species lacks early (primary) mesenchyme cells in the blastocoel, and pointed to the potential value of cidaroid developmental studies for understanding echinoderm phylogeny. Here I present a description of the larval development, larval form, metamorphosis, and early juvenile growth of *Eucidaris thouarsi* (Valenciennes), the eastern Pacific congener of the Atlantic *E. tribuloides*. These two species of *Eucidaris* are believed to have arisen from a common ancestor since the formation of the isthmus of Panama (Mortensen, 1928; Lessios, 1981). This study documents additional aspects of development in cidaroids that differ from developmental processes in euechinoids. Developmental and phylogenetic implications for all echinoids and for echinoderms in general are discussed.

Materials and Methods

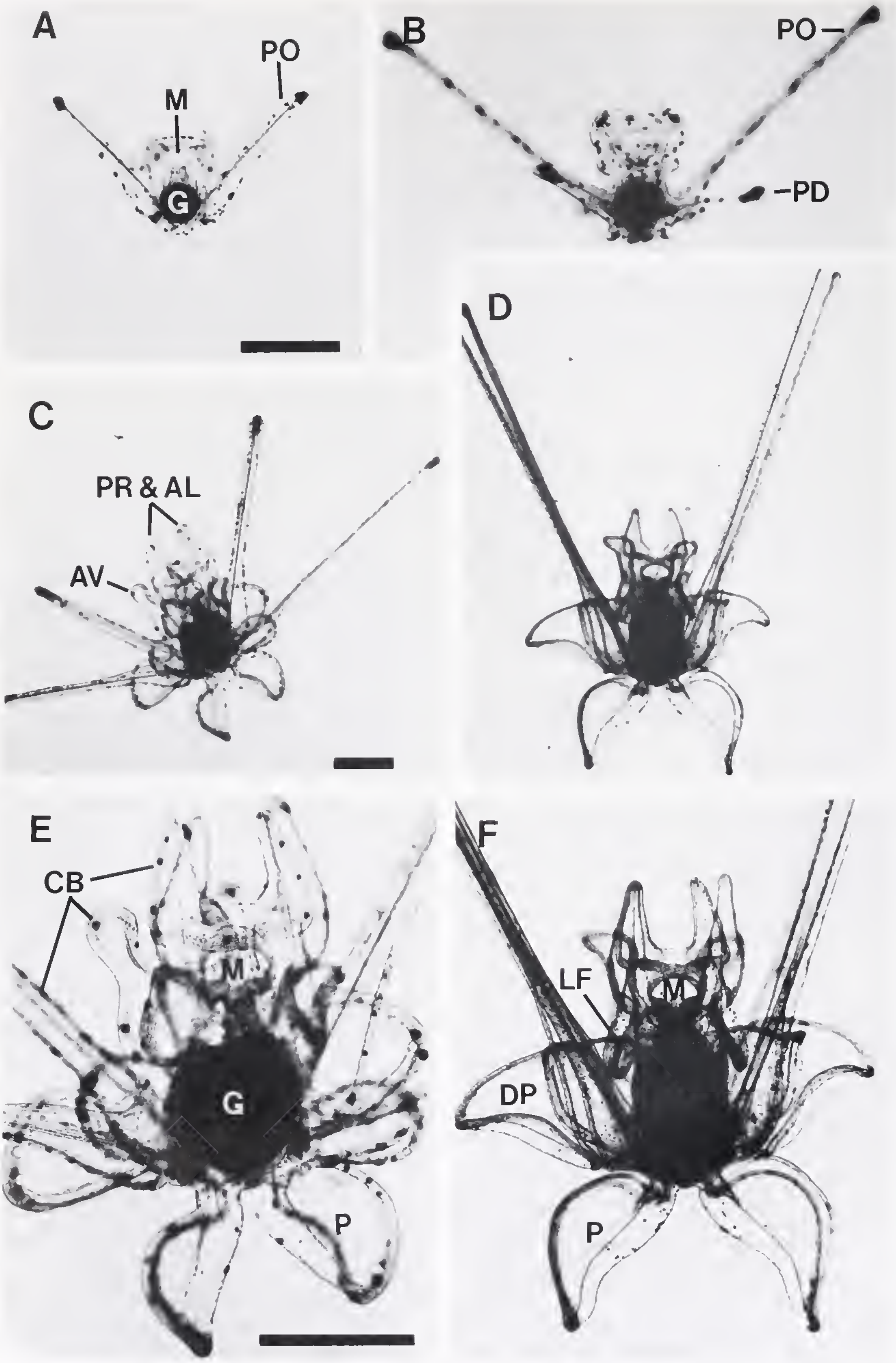
Adult specimens of *Eucidaris thouarsi* (Valenciennes) were collected at 3 to 5 m depth at Isla Taboguilla, Bay of Panama, Republic of Panama. Urchins were induced to spawn by intracoelomic injection of 5 to 10 mls of isotonic (0.55 *M*) KCl. Eggs were washed three times in filtered water (0.25 μm) and fertilized with several drops of a dilute sperm solution. Larvae were cultured in liter or gallon glass jars at a temperature of 28°C and salinity of 30‰ according to Strathmann's (1971) methods. Filtered water was changed on alternate days. Larvae were fed a combination of *Dunaliella tertiolecta* Butcher and *Rhodomonas lens* that were grown in a culture medium enriched with Alga-Gro Concentrate (Carolina Biological, Inc). Larvae were fed daily with algal cells that were first centrifuged and then resuspended in filtered seawater. Although no attempt was made to measure food concentrations, full larval guts indicated that adequate food was available. Terminology used for larval arms and spicules is from Mortensen (1921).

Observations of metamorphosis were made on late larvae with well-developed primary podia and juvenile spines. Larvae were put into small glass bowls with approximately 40 ml of filtered seawater. Each bowl contained a small piece of rock (1–2 cm^2) with organic (biological) films and encrusting algae (coralline and noncoralline red algae). Rocks were collected from adult habitats and held in the laboratory seawater table prior to use. Upon introduction to settlement bowls, larvae were observed for several minutes. They were then checked every 15 minutes for the next several hours to observe metamorphosis.

Larvae at different stages of development, including several that were metamorphosing, and several juveniles were fixed for 1 hour in buffered formalin (3%) and preserved in 70% EtOH. [Though other fixatives are superior to formalin, they were not available at the time the animals were preserved. It should be noted that the classic study by von Ubisch (1913) of the metamorphosis of the euechinoid *Paracentrotus lividus* was conducted on specimens fixed in buffered formalin. Furthermore, von Ubisch's results agree very well with studies on other euechinoids fixed in osmic acid and Müller's fluid (MacBride, 1903).]

Specimens from among these samples were prepared for observation on a scanning electron microscope (SEM) by critical point drying and coating with gold-palladium. Still other preserved specimens were prepared and serially sectioned according to the following methods. Specimens were decalcified in 5% EDTA (ethylenediaminetetraacetic acid) adjusted to pH 7.0 with NaOH, dehydrated through an alcohol series, and embedded in Spur embedding medium (Polysciences, Inc). Sections (3–5 μm thick) were cut in the frontal plane of the larvae, thus passing through the oral-aboral axis of the future juvenile. Sections were cut through juveniles in a similar orientation, the first sections passing through ambulacrum III. All sections were stained for 10 min with Harris' hematoxylin as modified by S. C. Chang (Smithsonian Institution), rinsed in running water, and counterstained for 10 min with basic fuchsin as modified by S. C. Chang. This staining protocol made it possible to identify nuclei, cytoplasm, some cytoplasmic granules, muscle cells, and collagenous connective layers.

The crystal orientations of larval spicules and apical plates were determined in late stage larvae and newly metamorphosed juveniles by observing them with polarized light on a microscope fitted with a Universal stage. The direction of the crystallographic axis was determined according to the methods of Emmons (1943). To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increase the transmission of light through soft tissues, specimens were first dehydrated and then put into unpolymerized embedding medium prior to observation under polarized light.

Results

Early development and larval form

The eggs obtained from 3 females had mean diameters of 87.7 μm (SD = 1.76, n = 25), 87.8 μm (SD = 4.70, n = 25), and 93.6 μm (n = 3). Early cleavage and development followed a pattern similar to that reported for *Eucidaris tribuloides* (Tennent, 1914; Schroeder, 1981). No mesenchyme cells were evident in the blastocoel until the gastrula stage when the archenteron extended halfway into the blastocoel. Spicules appeared by 28 h after fertilization. Larvae reached the two-armed stage and were feeding by 73 h after fertilization. Postoral arms grew laterally at first; as development continued, these arms became oriented more anteriorly but were still widely spread (Fig. 1A).

Table I summarizes observations on the development of two larval cultures of different parentage. The given times are observation times and do not necessarily represent the initiation of the reported events. In general, the variation in larval stages within cultures increased with time. At no time was there any indication that general culture conditions were poor or that culture conditions produced artifacts that might be misinterpreted in the results. Throughout the study when they were observed with dissecting and compound microscopes, larvae appeared to be healthy and progressing through developmental stages. After 30 days, when the first larvae metamorphosed, others had partially developed juvenile structures and still others showed no external sign of juvenile rudiment development.

The two-armed stage persisted through the 10th day after fertilization (Fig. 1A). During the development of the postoral spicules and arms, the calcareous rods associated with the anterolateral arms grew from the same spicules. These rods grew into the preoral hood region of the larva, but anterolateral arms that project anteriorly from the preoral hood region had not yet formed. Twelve to 15 days after fertilization, the posterodorsal arms formed and lengthened (Fig. 1B). By Day 15 some larvae had anterolateral arms, the dorsal arch spicule extended into the preoral hood region where "buds" of preoral arms were evident, and an unpaired posterior transverse spicule was present (Table I).

Morphology and behavior of late-stage plutei

From this point larval form began to depart markedly from the patterns typical of euechinoid larvae. Larvae of *Eucidaris thouarsi* developed epidermal lobes at specific locations on the body. Five pairs of lobes formed, all at locations along the ciliated band. Two pairs formed on each of the ventral and dorsal surfaces of the larval body; a fifth pair formed at the posterior end of the larval body, between the posterodorsal and postoral arms (Figs. 1C–F; 2A, C). Much of the rest of larval growth consisted of the lengthening of larval arms, including the anterolateral and preoral arms, and the enlargement of the epidermal lobes. A pair of lateral flaps extended from the preoral hood in a postero-ventral direction (Figs. 1D, 2A). Fully developed larvae had eight arms, five pairs of epidermal lobes, paired flaps on the preoral hood (Figs. 1D, 2A) and were approximately 2.5 to 3 mm long from posterior edge of the posterior lobes to the tip of postoral arms.

In larvae with developing lobes, the longer (postoral and posterodorsal) arms could be moved by muscles at the base of the spicules and posterior to the stomach (Figs. 3B, I). If the culture bowl was moved and the water disturbed, if the jar containing the larvae was tapped, or if larvae were subjected to suction from a pipette, larvae underwent a dramatic change in shape by moving the postoral and posterodorsal arms in unison from an anterior to a posterior orientation. Larvae flared these arms

Figure 1. Light micrographs of larval stages of *Eucidaris thouarsi*. All scale bars = 300 μm. A. Ten-day-old larva with a pair of postoral arms. B. Fifteen-day-old larva with two pairs of arms (same scale as A). C. Twenty-day-old larva with characteristic cidaroid lobes forming (ventral view). D. A ventral view of a twenty-nine-day-old larva with lobes highly developed but no juvenile structures (same scale as C). E. Higher magnification detail of C. F. Higher magnification detail of D (same scale as A).

Symbols used in Figures 1 through 4: *Larval arms and lobes:* AL, anterolateral arms; PD, posterodorsal arms; PO, postoral arms; PR, preoral arms; AD, antero-dorsal lobes; AV, antero-ventral lobes; DP, dorso-posterior lobes; VP, ventro-posterior lobes; P, posterior lobes; LF, lateral flap. *Other larval structures:* BL, blastocoel; CB, ciliated band; G, larval gut; H, left hydrocoel; LC, left posterior coelom; M, larval mouth, MU, skeletal muscles; Œ, esophagus; PT, posterior transverse rod; #, epidermis contracting along larval spicules. *Adult structures:* AS, adult spines; B, (podial) bud from water canal; C, perivisceral coelom; D, epidermis of oral disk; EL, epithelial layer; ES, epineural space; JS, juvenile spines; O, ocular plate; PB, primary podial bud; PC, epidermal pigment cells; PED, pedicellaria; RN, radial nerve; S, spine attached to genital plate 1; W, water ring; 1,2,3,4,5, genital plates 1–5.

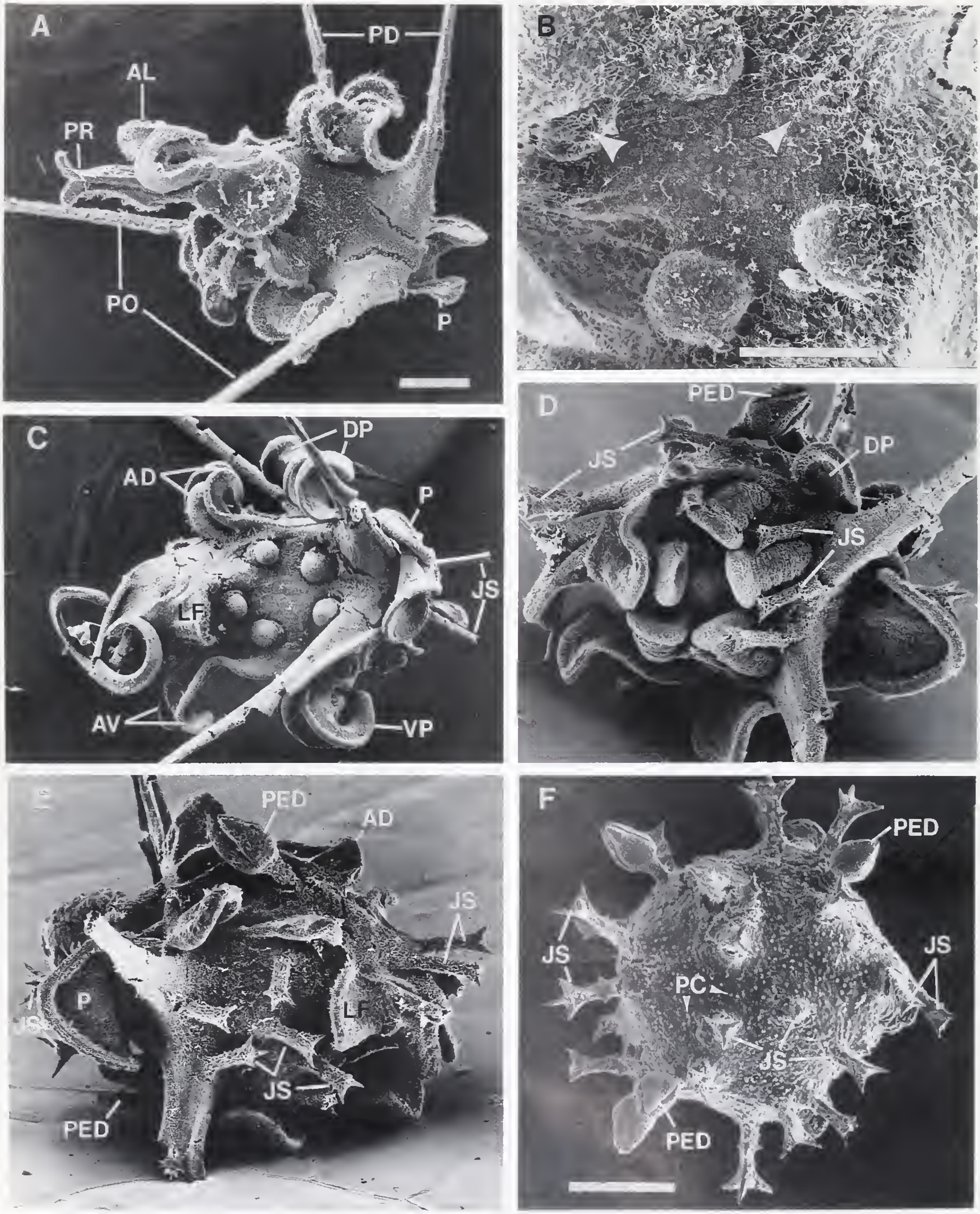
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Figure 2. Scanning electron micrographs of larvae of *Eucidaris thouarsi* A–D show progressive development of the primary podia which, in the absence of an amniotic sac, are exposed on the left side of the

Table I

Summary table of developmental events of Eucidaris thouarsi *(Valenciennes)*

Time since fertilization	Developmental stage or event
1.75 h	Four and eight cell embryos.
19 h	Early to mid gastrula. Archenteron extends 1/3 to 1/2 way into blastocoel. Mesenchyme absent.
21.5 h	Gastrula. Archenteron extended halfway into blastocoel. Mesenchyme is present. No spicules.
28 h	Late gastrula. Archenteron has contacted the blastocoel wall. Spicule primordia evident.
65 h	Prism/early two-armed larvae. Gut formation complete. Postoral spicules 115 μm long.
73 h	Feeding two-armed larvae. Stage is similar to that reported by Schroeder (1981, Fig. 3 O, P, Q).
10 days	Two armed larvae. Postoral arms well developed, 470 μm long (measured from posterior curve in ciliated band to arm tip).
11 days	Initiation of paired posterodorsal and unpaired dorsal arch spicules.
15 days	Posterodorsal arms are less than half the length of postoral arms. Buds of preoral arms and posterior transverse spicule are present.
18 days	Epidermal lobes are beginning to form.
20 days	Lobes continue to develop. Anterolateral and preoral arms grow anteriorly from the preoral hood.
25 days	Epidermal lobes are very highly developed. Postoral and posterodorsal arms approx. 2 mm long. The most advanced larvae have five primary podia, four pedicellariae, and two posterior juvenile spines.
27 days	More larvae have primary podia and pedicellariae, including one on the dorsal surface; juvenile spines are present in several locations on the larval body. Most advanced stages show reductions in arm length and lobes.
30 days	First larvae metamorphose. Juvenile test diameter approx. 510 μm.

Cultured larvae grew at 28°C and salinity 30 ppt.

outward and then backward, moving each arm through approximately 90 degrees of rotation. This flaring movement was rapid and temporary, with arms moved from the original position to the posterior position and back to the anterior position in approximately 1 s. Often the flaring reaction was repeated several times (2 to 5 or more times). During the flaring movement the larval body was thrust anteriorly while the arms moved posteriorly; as the arms returned to an anterior position the larval body moved posteriorly. After single or multiple flarings, there was no net movement of the larva. Larvae were never observed to use this flaring movement as a means of locomotion. Throughout development in culture bowls, larvae swam with their anterior ends upward. Maintaining this orientation, they moved slowly up or down with the aid of currents produced by the ciliated band.

Development of juvenile structures

The first juvenile structures to form were pedicellariae and embryonal or juvenile spines (Table I). A pair of pedicellariae formed at the bases of the posterodorsal arms on the dorsal surface of the larva. Another pair of pedicellariae formed at the bases of the postoral arms on the ventral surface. At the extreme posterior end of the larva, between these two pairs of pedicellariae, a pair of juvenile spines formed in association with the posterior transverse rod (Fig. 2C). No pedicellariae formed on this last rod. Several days later, a single pedicellaria and two juvenile spines formed on the dorsal surface of the larva over the region of the dorsal arch spicule (Fig. 2D).

As these structures were forming, buds of primary podia emerged on the left surface of the larval body (Fig. 2B, C). These buds were not enclosed in a vestibule or amniotic sac, but were exposed on the larval surface. Observation with SEM of the left surface of two- and four-armed larvae (not pictured) failed to show an invagination or cellular irregularity on the left surface that might indicate an amniotic invagination. Podia appeared first as bulges on the left surface (Fig. 2B). Over approximately 5 days, the podial buds grew into functional tube feet with terminal discs (Figs. 2C, D). After this time po-

larval body. Note also the lack of external evidence for epineural folds. A. Eight-armed larva with well-developed lobes, but without juvenile structures. Anterolateral arms folded during preparation for SEM (scale bar = 100 μm). B. A higher magnification of left surface of body region of an older larva shows the first external evidence of podial buds. Arrows indicate two of five buds that are less well developed (scale bar = 50 μm). C. Larva with podial buds at an intermediate stage of development (same scale as A). D. An advanced larva with primary podia that have well-formed terminal discs (same scale as A). E, F show an advanced larva from right side and a juvenile in the corresponding orientation (with interambulacr [illegible] on the left). E. Right side view of same advanced larvae as D. The two posterior pairs of larval arms have been broken off to allow orientation of the larva (same scale as A). F. A juvenile, one day after metamorphosis; the only spines present are juvenile spines. The corresponding spines and pedicellariae are marked in 2E and F and show that only small positional changes occur at metamorphosis (scale bar = 100 μm). See legend to Figure 1 for symbol identification.

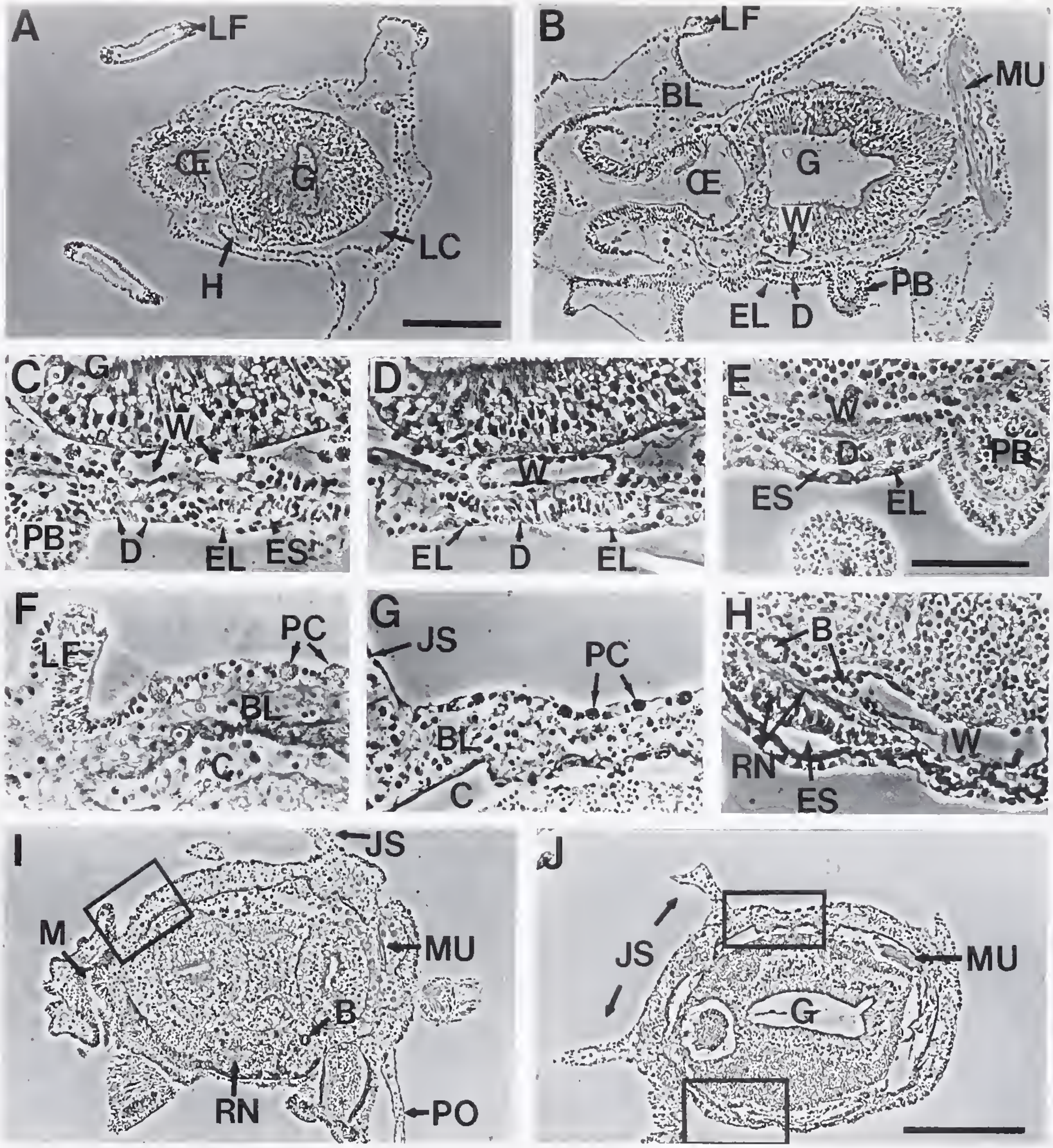

Figure 3. Histological sections of larvae and juveniles, phase contrast photomicrographs. All sections through larvae are frontal sections passing through the developing oral region of the juvenile. Each photograph is oriented with the larva's posterior (or corresponding part of the juvenile) on the right, and with the larva's left side (or juvenile's oral side) at the bottom. A. An early eight-armed larva with the hydrocoel as a simple sac on the left, anterior side of the gut (scale bar = 100 μm). B, C, D. An ordered sequence of sections through a larva with initial buds of primary podia and with epithelial layers partially covering the epidermis of the oral disk. B. A section, dorsal to the medial plane, passing tangentially through the water ring and through part of two podial buds. At the level of this section the epithelial layer covers the oral disc (same scale as A). C. Higher magnification of a section passing through a more medial region of the water ring (note 2 lumens are present and the labeled podial bud is ambulacrum II). The epidermis of the oral disk is exposed on the left but is covered by an epithelial layer on the right side (same scale as E). D. Section, ventral to the medial plane, passing tangentially through the water ring. At this location epithelial layers (and underlying epineural spaces) are on the left and right of the oral disk which is exposed in the middle of the photo, below the water ring (same scale as E). E. A later larval stage, with the oral disk completely enclosed by the epithelial layer (scale bar = 50 μm). F. A section through the epidermis of a competent larva. The approximate location of this section is indicated by the box in I (same scale as E). G. A section

dia were seen to move and eventually extend out from the larval surface by muscular contraction.

Histological sections through eight-armed larvae without podial buds showed the larval epidermis as a simple epithelium of cuboidal cells, and showed a pouch-shaped left hydrocoel located on the left side of the stomach (Fig. 3A). Before the podial buds began to form, the left hydrocoel developed into the ring canal, but the details of this process were not followed. By the time podial buds formed, the larval epidermis on the left side of the larval body was a stratified epithelium, several cell layers thick, and matched the description of the "ectodermic" or "oral disc" of euechinoids (Theel, 1902; MacBride, 1903). Together with hydrocoelic lobes that became the radial canals, the epidermis of the oral disc formed five evaginations that were the beginnings of the podial buds. Serial sections through larvae in early stages of podial bud formation showed a simple epithelium overlying the epidermis of the oral disc at its periphery and between the podial buds. At this stage, the center of the oral disc lacked this epithelial cover (Figs. 3B–D). The parts of this epithelium closest to the center of the oral disc were thin, had few nuclei, and appeared to be one cell thick. The epithelial cells tapered to an acute angle at their medial edges on the disc and here the epithelium lay flat on the epidermis of the oral disc (Figs. 3C, D). In this region the epithelium appeared to be continuous with the underlying epidermis of the oral disc, but could be distinguished from it by the orientations of the nuclei and by the staining properties of the cytoplasms. Peripheral to this thin layer, the epithelium was stratified into two layers, each one cell thick. In this location gaps between the epithelium and the epidermis of the disc opened, as did gaps between the two cell layers of the epithelium (Figs. 3C, D). The gaps between the epithelium and the epidermis of the oral disk correspond to the epineural space of euechinoids. In a slightly more advanced specimen (with longer podial buds) the epithelium completely covered the oral disc, however the podial buds remained free of this epithelium (Fig. 3E). In this specimen the epineural space between the epidermis and the epithelial layer was continuous but narrow across the center of the oral disc. A darkly stained connective tissue lay between the epithelial cell layers and there were few gaps between the epithelial layers (Fig. 3E).

At no time during the development of primary podia was there external evidence of the epithelium encroaching on the oral disc epidermis. SEM observations showed a complete, evenly ciliated and flat surface in and around the oral region (Fig. 2B). There was no indication of tissue folds forming lobes (with free edges) and moving into the region of oral disc, as has been reported in the formation of the epineural cavity of euechinoids (Theel, 1902; MacBride, 1903; von Ubisch, 1913; Hyman, 1955).

As the podial buds developed, the larval epidermis of the oral disc thinned in the center and thickened in areas around the ring canal and radial canals. Between the thickened regions of larval epidermis and the ring and radial canals of the hydrocoel, a region formed that appeared to contain fiberous material (Figs. 3E, H). The topographical arrangement of these tissues was similar to the description of the nerve ring and radial nerve given for the euechinoid *Paracentrotus lividus* by von Ubisch (1913).

In the most advanced larval stages, podia were well developed, and numerous juvenile spines could be seen on the dorsal, ventral, and right surfaces of the larvae (Fig. 2D, E). At least 19 juvenile spines were counted in the epidermis of the larva pictured in Figure 2E. In the larvae raised through metamorphosis, no definitive or adult spines were present on the larvae prior to metamorphosis (Figs. 2E, 3I). Advanced stage larvae also had shortened larval arms and greatly reduced epidermal lobes (Figs. 2D, E).

Histological sections through larvae that were judged capable of metamorphosis failed to show evidence of Aristole's lantern, adult spines, or their primordia. The lobes of the left posterior coelom extended interradially toward the oral disc, and were similar to the dental sacs reported in similar stages of regular euechinoids (Theel, 1902; MacBride, 1903; von Ubisch, 1913). The radial water canals showed small buds of additional podia subterminal to the well developed primary podia (Figs. 3H, I). The larval epidermis of the late larvae was consistently thicker than earlier stages and contained numerous large pigment-bearing cells (Figs. 3G, I).

Metamorphosis

Advanced larvae metamorphosed within 2 h after being introduced to a small bowl containing filtered seawater and rock substrata. Larvae at similar stages never

through juvenile aboral epidermis, three hours after metamorphosis. This section is an enlargement of the region indicated by the upper box in J (same scale as E). H. A section through the oral surface of juvenile three hours after metamorphosis. The approximate location of this section is indicated by the lower box in J (same scale as E). I. Section through a competent larva (same scale as J). J. Section through a newly metamorphosed juvenile. Comparison with I shows that only small rearrangements and resorption in place of larval structures is required to transform a competent larva into a juvenile (scale bar 200 μm). See legend to Figure 1 for symbol identification.

metamorphosed in the larval culture bowls; they appeared to react to the presence of the rock substratum. Larvae that swam over the rock would often sink and make contact with the rock. Upon contacting the rock, larvae would flare their arms and hold them in the posterior position. At this point the larvae would attach their podia to the substrate. This posture always preceded metamorphosis, but occasionally larvae were also seen to release from the rock and to begin swimming about the culture dish with arms again directed anteriorly.

When metamorphosis began it was accompanied by a waving movement of the posteriorly directed arms, followed by a contraction of the larval epidermis down the postoral and posterodorsal spicules (Fig. 4A). When the epidermis had retracted most of the way down these spicules, these four rods were severed at their bases. The straight portions of the rods fell away from the epidermis, and their bases remained on the right (aboral) and posterior (lateral) surfaces of the larva (juvenile). The next external changes to occur were the loss of the larval form by resorption of the remaining ridges and lobes, contraction of the epidermis from other larval spicules, erection of the juvenile spines, and a rounding up of epidermis to attain the shape of a juvenile echinoid (compare Figs. 2E, F).

Despite changes in shape, much of the larval epidermis in the body region remained intact and became the juvenile epidermis. There is both indirect and direct evidence for this. Indirect evidence that the larval epidermis became the juvenile epidermis is based on the topologies of the late larvae and the early juveniles. Both these stages bear numerous juvenile spines and pedicellariae, lined with larval epidermis. These structures act as surface markers and show that very little rearrangement of the surface took place prior to and within 1 day after metamorphosis (Figs. 2E, F). Direct evidence that much of the larval epidermis remained after metamorphosis is based on a comparison of histological sections through competent larvae and through juveniles (3 h after metamorphosis). Again, these sections (Figs. 3I, J) indicate that resorption of larval structures in place is all that occurred to transform the larva into the juvenile. A continuity of the epidermis between these two stages was evident from the large pigment-bearing cells interspersed among thick, cuboidal epidermal cells (Figs. 3F, G). Sections show numerous cells within the blastocoel; many of these are probably the degenerating remains of resorbed larval arms and lobes.

The final skeletal change that occurred during the shape rearrangements of the epidermis was a return of the bases of the postoral and posterodorsal spicules to the orientations they had when the arms were directed anteriorly. This return of the bases of the postoral and posterodorsal rods to their pre-metamorphic positions was verified by observation of larvae and juveniles under polarized light. The crystallographic axes (C-axes) of the calcite in the postoral and posterodorsal rods coincide with the long axes of the rods (see also Emlet, 1985). The orientations of the C-axes of the spicule bases indicated their return to their original positions. Because of this movement of the spicule bases, all of the larval skeletal elements that remained in the juvenile were in positions they had occupied prior to metamorphosis.

Observations of the metamorphosing larvae and of the early juveniles (within 5 h of the initiation of metamorphosis) under polarized light showed little change in the positions of the bases of larval spicules. (Fig. 4A–C). During the next 48 hours, skeletal (genital) plates covered the aboral surface by medial growth, not by migration, of the skeletal elements (Fig. 4C–F). In addition, adult spines formed and grew from interambulacral plates that formed after metamorphosis (Fig. 4E, F).

Discussion

Comparison of development among cidaroids

This study on the development of *Eucidaris thouarsi* supports Mortensen's (1937) conclusion that cidaroids have a larval form distinct from that of other echinoids. Larvae of *Cidaris cidaris* (Prouho, 1887), *Eucidaris metularia* (Mortensen, 1937), and *Prionocidaris baculosa* (Mortensen, 1938) have all been described and figured with characteristic lobes associated with the ciliated band. It is on this basis that Mortensen (1937) suggested that the Mediterranean larva pictured by Müller (1854) was that of a cidaroid, either *Stylocidaris affinis* or *Cidaris cidaris*. In addition, Mortensen (1937) stated that a larva he originally identified as *Astropyga pulvinata* (Mortensen, 1921, Pl. V, Fig. 7) was also a cidaroid, probably *Eucidaris thouarsi* (due to the locality—Bay of Panama). Though some euechinoid larvae do develop ciliated lobes (see below), only in cidaroid larvae are the lobes so numerous and well developed. The lobes give cidaroid larvae a more elaborate shape than most euechinoid larvae. The high degree of development of these fleshy lobes and the elaborate ciliated band are reminiscent of some asteroid (bipinnaria) larvae. An arm flaring behavior similar to that of *E. thouarsi* was described and figured for larvae of *Prionocidaris baculosa* by Mortensen (1938, Plate II, Fig. 2).

All cidaroids probably lack an amniotic invagination (or vestibule) on the left side of the larval body. Descriptions of the development of juvenile structures in feeding larvae of *Eucidaris metularia* (Mortensen, 1937) and brooded embryos of *Goniocidaris umbraculum* (Barker, 1985) stated that podia could be seen. Neither study mentioned presence or absence of an amniotic sac. The lack of a vestibule in *E. thouarsi* is correlated with the

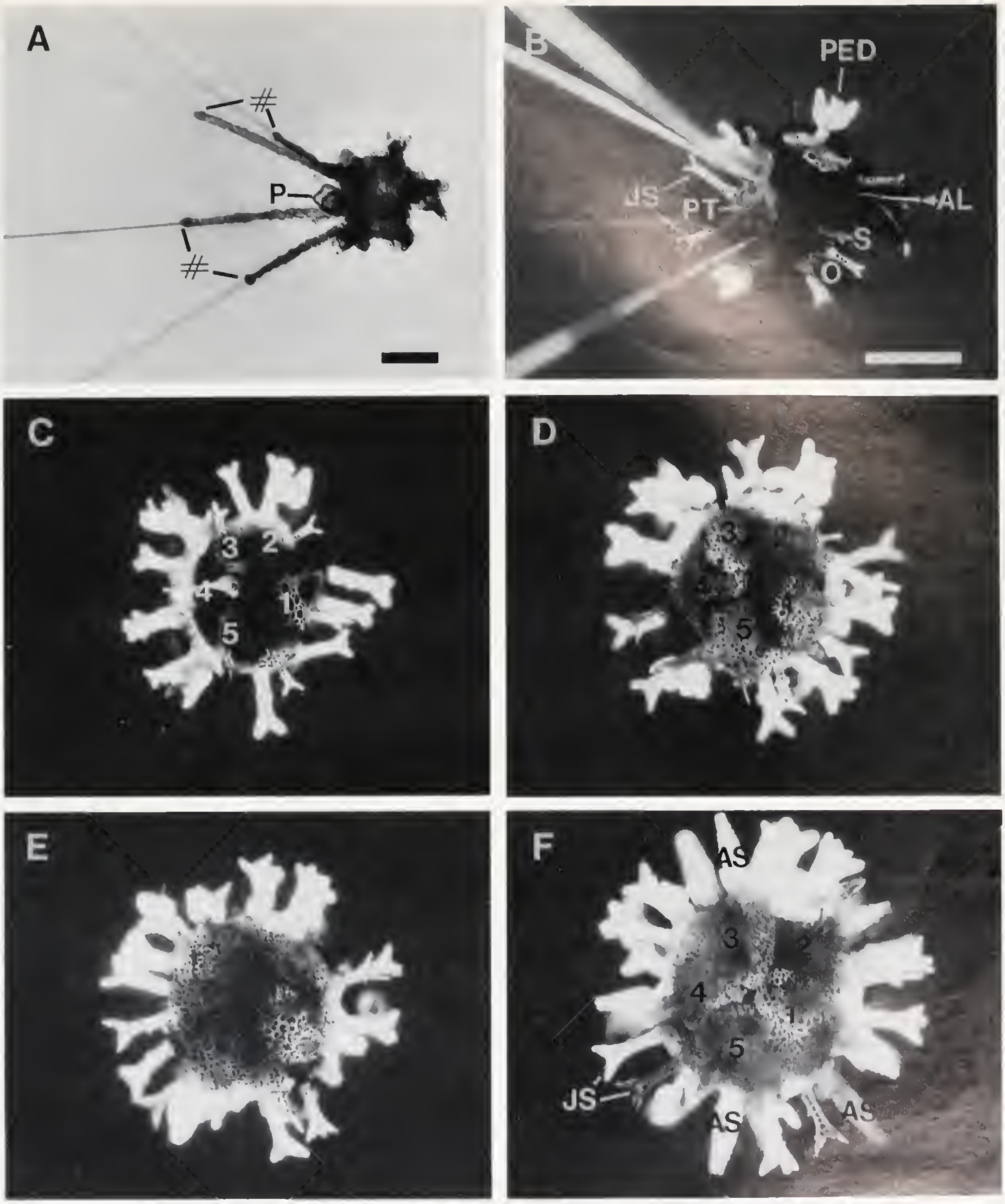

Figure 4. Light micrographs of a metamorphosing larva (A, B) and newly metamorphosed juveniles of *Eucidaris thouarsi* (C, D, E, F). All photographs are oriented with the posterior end of the larva and the corresponding part of the juvenile on the left side of the picture. Scale bars = 300 μm, B–F are all the same magnification. A, B. A larva during metamorphosis (right lateral view). B. Calcified larval and juvenile structures are revealed by polarized light. At the posterior end of the larva, two juvenile spines are attached to the posterior transverse rod. The bases of right postoral and posterodorsal rods are positioned at the periphery of the juvenile aboral surface to either side of a part of the posterior transverse rod. C. Juvenile 5 hours after metamorphosis. Numbers 1–5 indicate genital plates; four of these form from larval skeletal elements. D, E. Juveniles approximately one day after metamorphosis. The aboral surface of these juveniles is gradually being filled by the growth of apical plates. E is slightly more advanced than D. F. Juvenile, two days after metamorphosis. The apical system has almost completely filled the aboral surface. Adult spines can be seen at the circumference of the test and have a very different morphology from juvenile spines. See legend to Figure 1 for symbol identification.

numerous juvenile spines and absence of adult spines at metamorphosis. A similar morphology is seen in late larvae and early juveniles of *Prionocidaris baculosa* (Mortensen, 1938). Two cidaroids with large yolky eggs, *Phyllacanthus parvispinus* and *P. imperialis*, also lack signs of an amniotic invagination during modified development through a non-feeding larval stage (R. Raff, pers. comm.; R. Olson, pers. comm.). Because the euechinoid *Heliocidaris erythrogramma*, with non-feeding larval development has an amniotic invagination (Williams and Anderson, 1975), the absence of a vestibule in species of *Phyllacanthus* is probably a cidaroid trait and not due to modified development. The uniformity of morphology among feeding larvae of cidaroids supports my contention that the absence of a vestibule is typical of cidaroids.

McPherson's (1968) study of the biology of *Eucidaris tribuloides* included photos of a 20-day-old larva (Fig. 23) and a juvenile 15 days after metamorphosis (Fig. 24). It is unlikely that either of the photographed specimens are *E. tribuloides*. The larva pictured by McPherson lacks the highly developed lobes found on other cidaroid larvae including *Eucidaris metularia* and *E. thouarsi* (Figs. 1D, 3). Even McPherson acknowledged that "lobes were not as conspicuous as those described for *E. metularia* by Mortensen (1937)" (pg 430). The larva pictured by McPherson has a well-developed pedicellaria on the posterior transverse rod, but lacks the juvenile spines at the posterior end. Larvae of *E. thouarsi*, *E. metularia*, and *P. baculosa* always have a pair of spines at the posterior end. These larvae also have one pedicellaria at each of the bases of the posterodorsal and postoral body rods, but not on the posterior transverse rod. The similarity between cidaroid larvae in general, and especially between larvae of *Eucidaris metularia* and *E. thouarsi*, makes it unlikely that the larvae of *E. tribuloides* would differ in the ways reported by McPherson (1968). One larva in McPherson's culture metamorphosed and lived for 15 days. He noted that the juvenile had two types of spines and ambulacral structures resembling sphaeridia, and he remarked on their apparent absence in other cidaroids. The juveniles of *E. thouarsi* reared in the present study never grew structures resembling sphaeridia, and though juveniles had two types of spines, the adult spines differed in morphology from those of the juvenile pictured by McPherson (1968). The larva and juvenile pictured by McPherson (1968) appear to be those of a euechinoid, possibly *Echinometra* sp. or *Tripneustes* sp.

Comparisons of cidaroid and euechinoid larvae

Cidaroids and euechinoids differ in a number of ways throughout development (Table II). Raff *et al.* (1984) showed that euechinoids possess maternal α-subtype histone mRNA in their ova, while this mRNA is absent in the ova of cidaroids, asteroids, and holothurioids. Tennent (1914, 1922) and Schroeder (1981) emphasized other aspects of the early development of cidaroids that differ from euechinoids (Table II). Together with the studies on cidaroid development by Mortensen, the present description of the formation of juvenile structures and metamorphosis of *E. thouarsi* shows that cidaroids differ markedly from euechinoids in later development as well (Table II).

The five pairs of lobes found on cidaroid larvae are similar in construction, occur in the same positions, and therefore are probably homologous with the epidermal lobes on euechinoid larvae. The pairs of dorso-posterior and ventro-posterior lobes and the pair of posterior lobes are located in the same positions as the vibratile lobes found on arbaciid, echinometrid, toxopneustid, and some clypeasteroid larvae. The pair of antero-dorsal lobes are in a position similar to the anterodorsal lobes on larvae of arbaciids and some spatangoid echinoids. In these last named euechinoid groups, the anterodorsal and posterior lobes contain skeletal rods that branch from existing spicules. With spicules present, these lobes are long and narrow extensions of the ciliated band and are called extra larval arms (*e.g.*, anterodorsal and posterolateral arms of arbaciids and spatangoids). These comparisons between cidaroid and euechinoid lobes and arms indicate that all pluteus arms may have originated from lobes.

The antero-ventral lobes of cidaroid larvae are poorly developed in other echinoid larvae and usually are evident only as small protrusions or bends between the postoral arms. Similarly, the lateral flaps on the preoral hood region of cidaroids are present in some euechinoid larvae in a reduced form of small bends in the ciliated band (*e.g.*, Strathmann, 1971).

The absence of an amniotic invagination and sac is one of the most striking features of development of *Eucidaris thouarsi*. In all euechinoids with feeding larvae, the oral and adoral structures of the juvenile develop in an amniotic sac and do not occur externally on the left surface until or just prior to metamorphosis (Harvey, 1956; MacBride, 1903; von Ubisch, 1913). The amniotic sac first forms as an inpocketing on the left larval surface during the six-armed stage. This epidermal invagination enlarges, contacts the growing left hydrocoel, and together these tissue layers form the oral surface of the juvenile inside the amniotic cavity (Hyman, 1955). The only euechinoid species with feeding larvae that do not form an amniotic sac by invagination are several (and possibly all) temnopleurids, *Genocidaris maculata*, *Temnopleurus hardwickii*, and *T. toreumaticus* (von Ubisch, 1959; Fukushi, 1959, 1960). During the four-armed larval stage of these species, a mass of cells buds off by invagination from the ectoderm of the left surface

Table II

Differences in the development of cidaroid and euechinoid larvae

Cidaroids	Euechinoids
Hyaline layer is very thin. Consequently, blastomeres do not adhere closely during early cleavages, E.tr., P.b. (Mortensen, 1938; Schroeder, 1981).	Hyaline layer is relatively thick. Blastomeres are tightly adherent.
α-subtype histone mRNA absent from eggs, E.tr. (Raff *et al.*, 1984).	Maternal, α-subtype histone mRNA present in egg nucleus.
Two to three micromeres of variable size and equivalent numbers of macromeres, E.tr. (Schroeder, 1981).	Four equivalent sized micromeres and four equivalent sized macromeres.
No apical tuft forms after hatching, E.tr. (Schroeder, 1981).	Apical tuft present.
Slow development to pluteus, C.c. E.th., E.tr. (Prouho, 1887; this study; Tennent, 1914; Schroeder, 1981).	Development is faster in Lytechinus and Tripneustes (Tennent, 1914, 1922) and in other euechinoids (Amy, 1983; Emlet *et al.*, 1987).
Mesenchyme first forms at the tip of the archenteron mid way through gastrulation, E.tr., E.th. (Tennent, 1914; Schroeder, 1981; this study).	Primary mesenchyme appears on the vegetal plate prior to gastrulation, secondary mesenchyme appears in a manner similar to mesenchyme of cidaroids.
Location of initiation of skeleton is more medial, E.m., E.th., E.tr. (Mortensen, 1937; this study; Tennent, 1914).	Skeleton is initiated at the posterior end of the gastrula on the vegetal plate.
Shape of gastrula/prism stages is elongate, the organization of the ciliated band resembles the asteroid larva of *Asterias*, E.tr. (Tennent, 1914).	Gastrula is only slightly elongate. Prism is not asteroid like.
Formation of 5 pairs of epidermal lobes, E.m., E.th., P.b. (Mortensen, 1937, 1938; this study).	Many species possess epidermal lobes called vibratile lobes (2 pair) as well as other epidermal lobes, though never in the same high degree of development (see text).
Formation of lateral flaps on the preoral hood, C.c., E.th., P.b. (Prouho, 1887; this study; Mortensen, 1938).	Lateral flaps reduced or absent.
Podia grow directly out of the left surface of the larval body, E.th. (this study).	All feeding larvae studied have an amniotic sac inside of which podia and adult spines form.
Epineural space forms between an external epithelium and the epidermis of the oral disk. The juvenile morphology is similar to euechinoids, but the process may differ, E.th. (this study).	Epineural folds form between primary podia and fuse to create an epineural cavity. Described for several species Echinidae and one spatangoid, see text for references.
Metamorphosis is simple, no reorganization of the larval spicules or drastic contraction of the larval epidermis onto the right (aboral) surface of the larva (juvenile), E.th. (this study).	Metamorphosis involves eversion of the oral surface (podia and adult spines) through the vestibular opening and contraction of larval epidermis and larval skeletal elements to the right (aboral) surface of the larva (juvenile).
Much of larval epidermis retained by juvenile, E.th. (this study).	Larval epidermis is histolyzed. Juvenile epidermis from vestibular walls.
Numerous juvenile spines, E.th., P.B. (this study; Mortensen, 1938).	Few or no juvenile spines.
Absence of adult spines at metamorphosis, E.th., P.b. (this study; Mortensen, 1938).	Well-developed adult spines form around the juvenile oral surface within the vestibular cavity.

Traits of cidaroids are compiled from various species, identified by the following abbreviations: C.c., *Cidaris cidaris;* E.m., *Eucidaris metularia;* E.th., *Eucidaris thouarsi;* E.tr., *E. tribuloides;* P.b., *Prionocidaris baculosa.* Traits of euechinoids are generalizations from a large body of literature, including regular and irregular forms.

of the larva and migrates to a position where it contacts the left hydrocoel (von Ubisch, 1959; Fukushi, 1960). Where this mass of cells and the left hydrocoel contact, an amniotic cavity forms and podia and definitive spines of the juvenile develop, as in other euechinoids, enclosed in a cavity (Fukushi, 1960).

Within the amniotic sac of euechinoids (*Psammechinus miliaris,* Theel, 1902; *Echinus esculentus,* MacBride, 1903; *Paracentrotus lividus,* von Ubisch, 1913; *Echinocardium cordatum,* MacBride, 1918), folds of epidermal tissue form between the podia and grow toward the center of the juvenile oral surface. These "epineural folds" are described and figured as "free edges" raised above the floor of the amniotic sac or oral disc (Theel, 1902 plate I, sections 49, 50, & 51; MacBride, 1903 p. 304 and Figs. 40 & 41b; von Ubisch, 1913 Figs. 7 & 8). These folds join along their lateral edges and along their edges central to the oral disc. They enclose an epineural cavity external to the original floor of the amniotic sac. These epineural folds thus enclose the ambulacra and superficial cavity under a double layered epithelium (MacBride, 1903; Hyman, 1955). Sections through the developing oral disc region of euechinoids indicate that the double layered epithelium fits loosely over the epineural cavity, and there is often a large gap between the two epithelial layers (MacBride, 1903 Figs. 44 & 46; von Ubisch, 1913 Figs. 9 & 10). Mesenchyme cells that will form adult test plates migrate into the region between the double layers of epithelium. The epineural cavity and underlying oral disc tissue become restricted to areas along the ring canal and the radial canals of the hydrocoel (von Ubisch, 1913).

Enclosure of the ambulacra also occurs in *Eucidaris* *thouarsi*, but differs from euechinoids in the pattern by which the epithelial cells cover the oral disc region. The different patterns may represent different cellular mechanisms of tissue formation. In *Eucidaris thouarsi*, because the most medial region of the covering epithelium is very thin and in contact with the epidermis of oral disc, the epineural cavity appears to be formed by separation of the epithelium from the underlying epidermis. The more peripheral parts of the covering epithelium is double layered, but the space between these layers is never large and does not persist. The double layered epithelium does not appear to be loosely fitting. In *E. thouarsi* no external evidence of epineural folds was seen, nor did the external surface ever appear loosely fitting in the developing oral region. I know of no similar SEM observations on euechinoids, but von Ubisch (1913) presented schematic drawings of ambulacral enclosure which suggested that the epineural folds should be visible within the vestibule.

Observations of ambulacral enclosure in *Eucidaris thouarsi* are consistent with two different, potential mechanisms. An epithelium may migrate from between podial buds toward the center of the oral disc, its leading edge in contact with and "crawling" across the underlying epidermis. In this way, the leading edge drags along an epithelium that is doubled because one part of the epithelium remains anchored at the periphery of the oral disc. After the leading edge passes a location, an epineural space forms by delamination of the epithelium and the underlying epidermis. Alternatively, the epithelium with underlying cavity could form by the spreading of separate cavitation sites that begin peripherally and move toward the center of the oral disc. In the older regions of separation of the two tissues, the superficial epithelium has divided again to form two layers. Detailed ultrastructural work on *both* groups of echinoids is required to distinguish between these two possible mechanisms of ambulacral enclosure and determine how the mechanisms of ambulacral enclosure may differ between cidaroids and euechinoids.

My observations of *Eucidaris thouarsi* on the formation of the radial canals and of secondary podial buds, on the formation of the nerve ring and the radial nerves from oral disc tissue, and of interradial ingression of the left posterior coelom to form dental sacs were similar to those reported for regular euechinoids (von Ubisch, 1913).

Few embryonal or juvenile spines form on the epidermis of euechinoid larvae (*e.g.*, Fukushi, 1960; Onoda, 1931; Emlet, unpubl. obs.). Usually these spines have a characteristic structure with three or four points at their tips, and grow at the posterior end, on the dorsal surface (near the dorsal arch), or on the right side of the larval body (near posterodorsal and postoral spicules). Larvae of certain euechinoid groups (*e.g.*, arbaciids, spatangoids, and clypeasteroids) lack juvenile spines on the larval surface altogether. In contrast, larvae of *Eucidaris thouarsi* possessed approximately 20 juvenile spines on the larval exterior prior to metamorphosis. Mortensen's (1938) figures of late stage larvae and newly metamorphosed juveniles of *Prionocidaris baculosa* indicate similar large numbers of juvenile spines for this species as well.

Advanced larvae of all euechinoids possess definitive or adult spines growing around the adoral juvenile surface within the amniotic cavity. (In *Arbacia* spp. these spines are spatulate rather than columnar.) Definitive spines were absent from newly metamorphosed juveniles of *Eucidaris thouarsi* and *Prionocidaris baculosa* (Mortensen, 1938). The absence of definitive spines in cidaroids at metamorphosis suggests heterochrony in spine formation and may be developmentally correlated with the lack of an amniotic cavity (see below).

In euechinoid metamorphosis, the eversion of the rudiment and contraction of the larval epidermis brings larval spicules and tissues to the aboral surface of the juvenile (right side of larva) and exposes the oral surface and definitive spines. Most of the larval epidermis on the aboral surface is resorbed and histolyzed, and the tissue of the vestibular walls becomes the juvenile epidermis (Bury, 1895; MacBride, 1903; Cameron and Hinegardner, 1978; Chia and Burke, 1978). The very different metamorphosis of *Eucidaris thouarsi* does not involve as drastic a rearrangement of larval tissues and skeletal elements. In cidaroids, external juvenile structures develop on the larval body. No major rearrangements of larval skeletal elements are required to establish juvenile morphology. Only slight movements accompany considerable growth of the bases of larval spicules to set up aboral (apical) plate arrangements in the juvenile. With no amniotic sac and sac lining in cidaroids, the larval epidermal lobes and arms are resorbed in place into the juvenile surface, and the rest of the larval epidermis remains intact to become juvenile epidermis.

Implications for development

This study shows that development and metamorphosis of feeding larvae varies among echinoids and can no longer be generalized from the early descriptions of euechinoid (Echinidae) development by Bury (1895), MacBride (1903), and von Ubisch (1913). Mortensen (1921, p. 230) warned against considering the larvae of the Echinidae to be generally representative of the echinoids, but his advice has largely gone unheeded. Raff (1987) argued that development of feeding larvae of echinoids is highly constrained relative to development of nonfeeding larvae. To the extent that direct developing larvae show greater variation in the timing of initiation of adult structures, this may be true. However, this study along with the above mentioned studies on temnopleuroids indicates that considerable variation in timing and mode of formation of the amniotic sac also occurs in feeding echinoid larvae. This flexibility within the Class is distributed along taxonomic lines.

The differences between cidaroid and euechinoid development indicate that different developmental pathways can be taken to reach the same objective: a juvenile sea urchin. The lack of an amniotic sac in cidaroids has no lasting effect on the post metamorphic juvenile, but has a profound affect on the complexity of metamorphosis and on the fate of some larval and early juvenile structures. With an amniotic sac metamorphosis is relatively complex; without it, metamorphosis is simpler. The amniotic sac of euechinoids is important in metamorphosis because it is the site of formation of definitive structures, such as adult spines and epidermis. In cidaroids the absence of a vestibule is correlated with a different fate for the larval epidermis and with the absence of adult spines. The absence of an amniotic sac may also account for some of the differences reported here on the enclosure of the ambulacra. Inside an amniotic sac, podial buds are forced to grow toward the center of the oral disc. Euechinoid epineural lobes may be exaggerated by being restricted to areas between podial buds which are crowded together by the roof of the amniotic sac.

The differences in spine formation between euechinoids and cidaroids indicate that some compensation by different developmental processes may be occurring. The presence in cidaroids of a large number of juvenile spines may compensate for the lack of definitive spines at metamorphosis. Conversely, presence in euechinoids of definitive spines within the amniotic sac may reduce or eliminate the need for juvenile spines at metamorphosis. The delayed formation of adult spines in cidaroids is another example of differences in timing of developmental events. In euechinoids, an amniotic sac with its lining of adult epidermis may permit the precocious formation of definitive spines around the oral surface of the juvenile that might otherwise interfere with normal functions of swimming and feeding by the pluteus. Strathmann (in press) discussed numerous other developmental events of echinoderms that may not be independent.

Implications for phylogenetic studies

The amniotic invagination must now be considered both present and absent in the class Echinoidea. The absence of an amniotic invagination and the pattern of ambulacral enclosure in cidaroids raises further questions about the utility of certain ontogenetic characters in establishing relationships between Classes and about the origin of the amniotic sac of euechinoids. The characters described for cidaroid larvae could readily be interpreted as primitive among echinoids, but polarizing them as such requires comparison with a sister group to the echinoids. Regardless of the choice of sister group, convergence of developmental characters among the classes occurs frequently (see Strathmann, in press).

Though relationships between classes of echinoderms are still uncertain, several recent phylogenies of echinoderms have echinoids and holothuroids as sister groups (Smith, 1984b; Raff *et al.*, in press) or echinoids and ophiuroids as sister groups (Smiley, in press; but also see Strathmann, in press). Both of these possible choices for sister groups to the echinoids develop the adult mouth at the site of the larval mouth. In contrast, echinoids and asteroids develop the adult mouth on the left side of the larval body, independently of the larval mouth. The present study suggests cidaroid echinoids and asteroids are even more similar in development, because both lack epidermal invaginations at the site of formation of the adult mouth. The great differences in adult morphology and other differences in larval morphology make it unlikely that these striking similarities between asteroid and cidaroid larvae are due to a shared ancestor, but rather due to morphological convergence.

The description of ambulacral enclosure given here for *Eucidaris thouarsi* is quite similar to that described for the ophiuroid *Ophiopholis aculeata* (Olsen, 1942). In describing the epithelial tissue that covered the oral epidermis, Olsen (p. 81) stated that the inner layer of the double layered tissue was at first in contact with, but later separated from, the underlying epidermis. The cavity that formed was the epineural canal. Earlier studies on ophiuroid development (MacBride, 1907; Narasimhamurti, 1933) report epineural folds enclosing the ambulacra but are not clear on the details of the relationship of epithelial and underlying epidermal tissue layers. Since epineural folds were first observed in ophiuroids, they have been considered homologues of the epineural folds in euechinoids (MacBride, 1907; Hyman, 1955). If the description of ambulacral enclosure for *Eucidaris thouarsi* generalizes to all cidaroids and the description for *Ophiopholis aculeata* generalizes to all ophiuroids, the similarity may reflect common origin. This similarity may also be convergent and due to the open surfaces on which these processes are occurring in ophiuroids and cidaroids.

The term *vestibule* refers to an epidermal invagination on the larva at the site of the adult mouth, around which the water vascular system develops (Ubaghs, 1967). The implicit function of the vestibule is to bring epidermal tissue into contact with hydrocoelic tissue to initiate formation of adult oral structures. This term is used to describe the oral cavity of holothuroid and ophiuroid larvae because the left hydrocoel of these larvae grows around the esophagus just beneath the epidermis of the oral cavity (Smiley, 1986; Olsen, 1942). The highly modified, non-feeding larvae of crinoids possess an invagination identified as a vestibule, but its relationship to larval structures is uncertain. An indication that this vestibule is a vestige of the larval mouth is implied by Lacalli and West's (1986) study of transitional patterns of ciliation in the crinoid larva of *Florometra serratissima*. The same term is used to describe the amniotic invagination and sac on the left side of the larval body of euechinoids. Because of the involvement of these epidermal invaginations in formation of the adult mouth, Hyman (1955) suggested they were homologous across the phylum. Hyman's view of homology of vestibules ignores the fact that the amniotic sac of euechinoids is not associated with the larval mouth but is located on the left side of the larval body. The vestibule of other classes and the amniotic sac of euechinoids also differ morphologically because this later invagination is completely sealed off from the exterior during a portion of development. Based on positional and morphological criteria, the amniotic sac of euechinoids should not be considered homologous with the vestibule of other echinoderms. This conclusion is paradoxical, because the adult mouths of extant echinoderms are considered homologous.

There is another line of evidence that suggests the euechinoid amniotic sac is not homologous with the vestibule of other classes. Larvae from the four non-echinoid echinoderm classes and probably all cidaroids retain much of the larval epidermis to form adult epidermis (Chia and Burke, 1978). In contrast, euechinoids histolyze much of the larval epidermis and replace it with adult epidermis that originates from the amniotic sac. The similar fate of the larval epidermis in non-echinoid classes and cidaroids implies that the condition found in euechinoids is derived and is permitted by the amniotic sac. This line of evidence suggests that the euechinoid amniotic invagination is a derived character within the echinoids and that its absence in cidaroids is primitive among the Echinoidea.

If the absence of an amniotic sac in cidaroids is primitive among echinoids, the amniotic sac of euechinoids is either an independently evolved structure or an example of incomplete evolutionary reversal. Similarities between the amniotic sac of euechinoids and the vestibules of other echinoderms suggest that an evolutionary reversal is more likely. A functional role for the amniotic sac of euechinoids as a site for precocious formation of adult epidermis, adult spines, and juvenile rudiment is compatible with this origin.

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Ophiuroid Skeleton Ontogeny Reveals Homologies Among Skeletal Plates of Adults: A Study of *Amphiura filiformis, Amphiura stimpsonii* and *Ophiophragmus filograneus* (Echinodermata)

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Abstract. The characteristic oral papillae of the Family Amphiuridae are shown to have conservative patterns of ontogenesis, even among congeners with differing modes of reproduction such as *Amphiura stimpsonii*, a brooder, and *A. filiformis* which has free-living juveniles. Homologous oral papillae can be identified by tracing the distinctive ontogenetic transformations of individual skeletal elements. This method shows that the oral papillae of adults are not serially homologous, and that homologies cannot necessarily be inferred from the relative positions of papillae in any particular ontogenetic stage. For example, the most proximal oral papilla develops like a tooth on the dental plate and later moves to the proximal oral plate; a distal papilla grows as a spine on the adoral shield and moves to the distal oral plate. Based on the development of the oral papillae of *Amphiura, Amphioplus,* and *Ophiophragmus* species, it appears that post-larval ontogenesis of the amphiurids can be more reliable than larval morphology as an indicator of phylogenetic affinity. However, there are striking differences in postlarval skeletal ontogenesis among congeners, such as in formation of the adoral shield spines and primary plates of the disc, which may be related to modes of reproduction and postlarval biology. It can take over a year for adult oral armature to develop in free-living amphiurid juveniles, and the process occurs before hatching in brooded young. Specializations in the oral armature of postlarvae are probably critical to their survival.

Introduction

The relationship between ontogenetic patterns and phylogenetic history has been a matter of debate for two centuries (Mayr, 1982). Putative cases of "recapitulation" (*sensu* Alberch *et al.*, 1979) attributed to shared pathways of development are at variance with instances where ontogenesis departs from an explicit recapitulation of ancestral characters (Alberch, 1985). These contradictory observations are "matters of current discussion" (Mayr, 1982: 476).

The larval development of echinoderms provides numerous examples of departures from recapitulation (see Fell, 1948; Strathmann, 1974) such as through "cenogenesis" (*sensu* Gould, 1977)—the introduction of adaptations that are expressed only during early developmental stages. Yet, systematists continue to use ontogenetic patterns as indicators of systematic relationships in extant and fossil echinoderms (for example, see McNamara, 1986).

The larvae and embryos of closely related ophiuroids can be strikingly different; some inconsistencies between larval morphologies and adult classification are thought to result from adaptations to the larval (or embryonic) environment (Fell, 1948, 1967). Therefore, one might ask whether the ontogenetic features of postlarvae can be more reliable indicators of systematic relationships than the features of larvae. To answer that question, I compared growth series of congeners with dissimilar modes of reproduction: *Amphiura filiformis* (O. F. Müller, 1776) with pelagic ophiopluteus larvae and *A. stimpsonii* Lütken, 1859, with brooded embryos (early developmental stages described in Mortensen, 1920, 1921). Can comparisons of growth series also be used to distinguish homologous structures in closely related taxa by tracing their postlarval ontogenesis? To answer that question the ontogenesis of the oral papillae in representatives of the four major groups (*sensu* Clark, 1970) of Amphiuridae

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Table I

The sequence of development of oral papillae in ophiuroid species representative of major groups of Amphiuridae

	Stage 1					Stage 2					Stage 3					Stage 5					Stage 6					Stage 9				
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
A. stimpsonii		+					+		+		+	+		+		+	+		+		+	+		+		+	+		+	
A. filiformis		+					+		+		+	+		+		+	+		+		+	+		+		+	+		+	+
O. filograneus		+					+		+		+	+		+		+	o		+		+	o	+	+		+	o	+	+	
O. scabriuscula		+					+		+		+	+		+		+	o		+		+	o	+	+		+	o	+	+	
A. squamata		+					+		+		+	+		+		+	o		+		+	o	+	+		+	o	+	+	
A. abditus		+					+		+		+	+		+		+	+		+		+	+	+	+		+	+	+	+	+
A. macilentus		+					+		+		+	+		+		+	+		+		+	+	+	+		+	+	+	+	+

Amphiura-group: *Amphiura stimpsonii, A filiformis Amphiodia*-group: *Ophiophragmus filograneus, Ophiocnida scabriuscula; Amphipholis*-group: *Amphipholis squamata; Amphioplus*-group: *Amphioplus abditus, A. macilentus.* Ontogenetic stages are identical to stages in Table I. Oral papillae are listed in their order of attachment from the tip to the base of the jaw: A, infradental papillae; B, buccal scales; C, oral plate papillae; D, adoral shield spines; E, accessory papillae. Oral papillae present are denoted "+"; resorbed oral papillae denoted "o."

are analyzed. The oral papillae are small ossicles (squamous, papilliform, or spiniform) attached to the edges of the plates around the ophiuroid mouth, and they are the most critical characters employed in the taxonomy of the Amphiuridae (Clark, 1970). The species compared are *Amphiura stimpsonii* and *A. filiformis* [*Amphiura*-group], *Ophiophragmus filograneus* Lyman, 1875 and *Ophiocnida scabriuscula* (Lütken, 1859) [*Amphiodia*-group], *Amphipholis squamata* (Delle Chiaje, 1828) [*Amphipholis*-group], and *Amphioplus abditus* Verrill, 1871 and *Amphioplus macilentus* Verrill, 1882 [*Amphioplus*-group] (Hendler, 1978; this report). I also assess the value of homologous oral papillae as ophiuroid systematic characters.

Further, one might ask whether differences in morphology between homologous structures are related to their function in various ontogenetic stages. Therefore, the adaptational significance of oral papillae are considered and the reliability of juvenile morphology as an indicator of developmental mode and substrate specificity are evaluated thus augmenting reports by Turner (1974), Hendler (1975), and Muus (1981). Information on the taxonomic features, functional morphology, and natural history of ophiuroid juvenile stages critical to an understanding of development and evolution can also be directly applicable to studies of ophiuroid reproductive biology, distributions, and population dynamics (Gage and Tyler, 1982; Hendler and Littman, 1986).

Materials and Methods

The 13 individuals of *Amphiura filiformis* examined using scanning electron microscopy (SEM) were collected from the Oresund, Denmark (Muus, 1981). Adult and free-living juvenile *A. stimpsonii* were collected from the Belize Barrier Reef (Hendler and Littman, 1986), and over 40 SEM preparations were made of embryos dissected from the bursae of brooding females. The eight juvenile specimens of *Ophiophragmus filograneus* from Tampa Bay, Florida, examined using SEM were originally collected and studied by Turner (1974). Adult *O. filograneus* were collected by the author from the Indian River, off Fort Pierce, Florida. The restricted numbers of specimens examined preclude a precise delimitation of the range of body size at each developmental stage. This does not affect the validity of the sequences of stages that are reported, but it confounds comparisons between the developmental stage and body size or age of different species.

SEM samples of *O. filograneus* were air-dried alcohol preserved specimens that were mounted on stubs using Bakelite glue and sputter coated with carbon and gold-palladium. Since the edges of many skeletal plates were obscured by integument in these preparations, soft tissue was removed from specimens of *Amphiura filiformis* and *A. stimpsonii* using a plasma-asher ("Plasmod" with pyrex chamber, March Instruments, Concord, California). Air-dried ophiuroids, held in glass containers, were ashed for 1–3.5 h before mounting them on SEM stubs. They were removed from the asher periodically to insure that the treatment did not detach skeletal elements.

Terminology and abbreviations

Two to five oral papillae are attached to each edge of an amphiurid jaw. Proceeding proximally from the tip of the jaw each oral papilla is specified by a different name as follows: infradental papilla, buccal scale, oral plate papilla, adoral shield spine, and accessory papilla. Terminology used is based on accepted names of structures in adult ophiuroids (Hendler, 1978). However "buccal scale" is used rather than "oral tentacle scale" (*sensu* Clark, 1970) for reasons previously explained (Hendler, 1978). "Oral plate papilla," used here, replaces

Table II

The body sizes of Amphiuridae at different ontogenetic stages

	Amphiura stimpsonii		*Amphiura filiformis*		*Ophiophragmus filograneus*		*Amphioplus abditus*		
Stage	AS	dd	AS	dd	AS	dd	AS	dd	Ontogenetic transformation
1	ι	0.4	?	#	?	#	55 h	larva	B appears.
2	ι	0.4	ι	0.3	1	0.5	ι	0.3	D appears.
3	7	0.5	9	0.7	14	1.4	5	0.7	A appears.
4	7	0.5	25	0.8	14	1.4	9	0.9	D points proximally.
5	***********		**********		55	2.4	**********		B resorbed.
6	***********		**********		55	2.4	17	1.1	C appears.
7	19	1.2	25	0.8	55	2.4	17	1.1	D migrates to distal oral plate.
8	19	1.2	?	3.2	55	2.4	21	1.3	Positive allometric growth of oral plate, adoral shield, oral shield; movement of ventral arm plate.
9	***********		**********		?	?	>30	1.6	E appears.

Symbols: AS, number of arm segments; dd, disk diameter in mm; ι, terminal arm plate; ?, data not available; *****, specified ontogenetic transformation omitted in the species; A, infradental papillae; B, buccal scales; C, oral plate papillae; D, adoral shield spines; E, accessory papillae; #, smallest specimens examined are Stage 2 with B- and D-papillae.

the potentially misleading designation "third oral papilla" (Hendler, 1978). The size of ophiuroids is expressed as the diameter of the disk ("dd") and the number of arm segments ("AS"). Arm segment number is counted from the first arm segment under the disk to the distalmost segment of the arm, excluding the terminal plate at the arm tip. In this report dd is the primary indicator of body size. Although the number of AS varies among individuals of any dd, only the maximum number of AS are presented for any given dd.

Results

Special features of each species are discussed below, and the major ontogenetic changes of the oral skeleton of *Amphiura stimpsonii*, *A. filiformis*, and *Ophiophragmus filograneus* are illustrated in Figures 2–4 and summarized in Table I. The size (dd and AS) of different developmental stages of the three species, and of *Amphioplus abditus*, are compared in Table II.

Amphiura stimpsonii *Lütken*

In the youngest individual examined the rudimentary skeletal elements form a fragile crust near the surface of the large yolk mass (Fig. 1A). During development, the disk diameter and number of arm segments increase and the yolk mass is overgrown and hidden by the disk. The largest brooded embryos found were 0.7 mm dd with 10 AS. Young may be released at a smaller size (some free-living juveniles are only 0.6 mm dd with 8 AS) but most small, free-living juveniles were 0.8 mm dd with 10 AS. The largest adult specimen examined with SEM (4.04 mm dd) had arms broken at 63 segments beyond the disk.

The earliest stage examined was 0.42 mm dd, with arms composed of only a terminal plate (Figs. 1A, 2A; Tables I, II). The oral frame consists of adoral shields, and of paired jaw plates that bear a dental plate and tooth. The rudiments of two oral papillae, the buccal scale and adoral shield spine, are inconspicuous; the adoral shield spine was found in only one of three specimens at this stage. A buccal scale is present in all specimens, and presumably develops before the adoral shield spine (Tables I, II).

The final oral papilla to develop is the infradental. A rudiment of the infradental papilla occurred at the tip of the jaw in a specimen 0.46 mm dd with 7 AS. Initially, the infradental papilla lies in an indentation on the dental plate, not on the jaw plate itself (Fig. 2B). The dental and oral plates are relatively larger than in the previous stage; they have grown ventral to the lowest tooth (out of the plane of Fig. 2C). At this stage and in newly hatched specimens approximately 0.7 to 0.9 mm dd with at least 10 AS, allometric growth transforms the oral frame. As a result of the enlargement of the oral shield and growth and divergence of the adoral shields, the adoral shield spine moves to a more proximal, adradial position on the adoral shield and it projects over the pore of the second buccal papilla rather than over the periphery of the disk (compare the orientation of the adoral shield spine [d] in Figs. 1A, 2A, C).

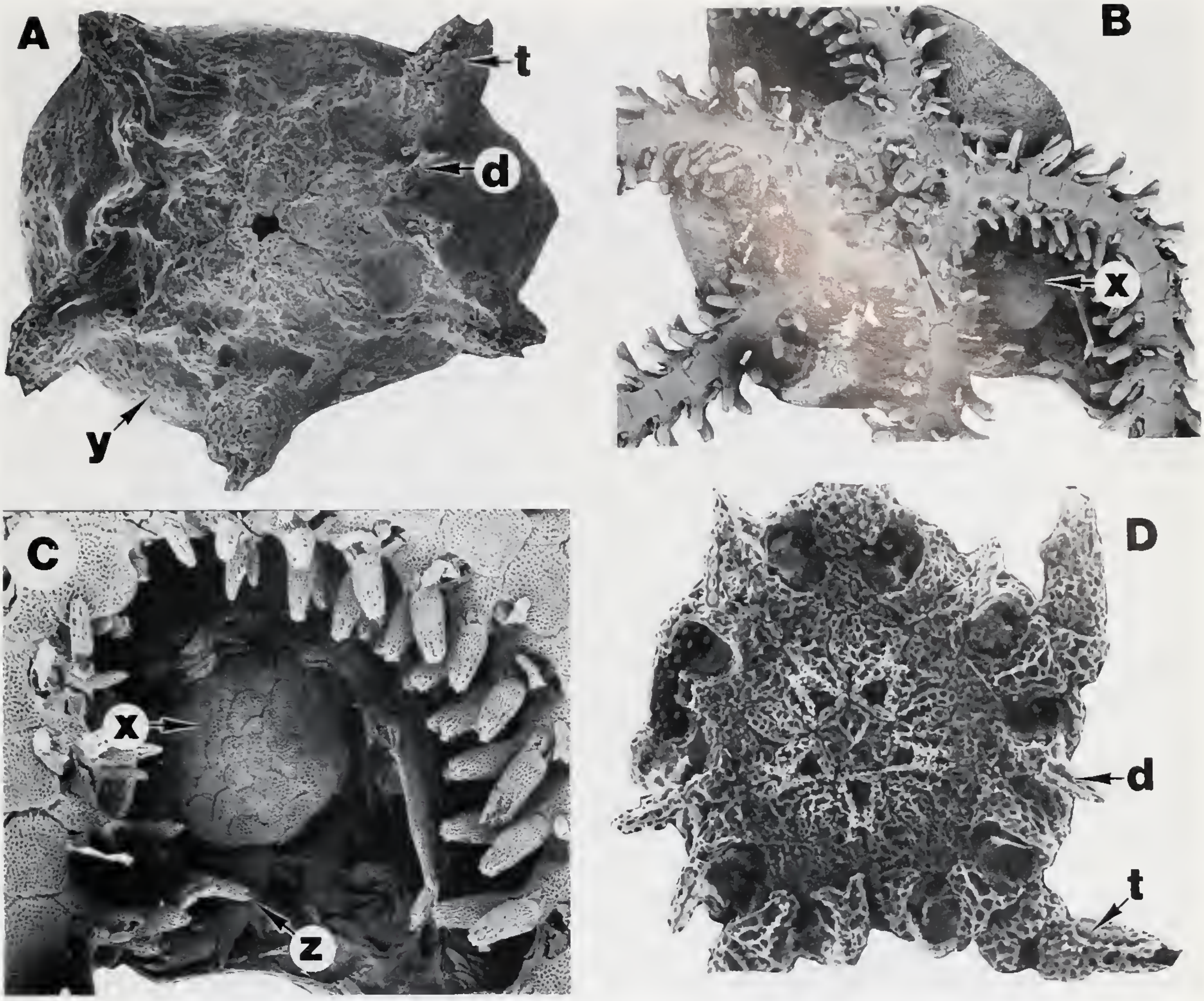

Figure 1. (A) *Amphiura stimpsonii:* early (Stage 2) unhatched embryo 0.42 mm dd showing large yolk mass and protruding terminal arm plate and small adoral shield spine. Specimen not plasma-ashed to remove integument. (B, C) *Amphiura stimpsonii:* ventral interradial body wall removed to show advanced brooded embryo in the bursa. Note gaps between jaws of adult (double arrowhead in B) and lack of primary rosette on disk of embryos (in C). (D) *Amphiura filiformis:* newly settled (Stage 2) juvenile 0.33 mm dd, with relatively large adoral shield spine. Plasma-ashed specimen. Abbreviations: d, adoral shield spine; t, terminal arm plate; x, disk of embryo; y, yolk mass; z, arm of embryo.

In a specimen 1.2 mm dd with 19 AS (Fig. 2D) the adoral shield spine moves from the adoral shield to the "distal oral plate" (*sensu* Hendler, 1978) that is, distal to a suture line (visible in Fig. 2C) that bisects the jaw. This remarkable transition is a consequence of marked allometric growth of the oral and adoral shields in specimens 0.86 to 3.76 mm dd (and probably of the oral plate muscle-attachment surfaces as shown for *Amphioplus abditus* in Hendler, 1978: Fig. 6). Due to differential growth rates of these jaw elements, the first ventral arm plates move from a superficial to an internal position on the disk, and the proximal end of the plate sinks into the oral gap (not shown in Fig. 2, but see Hendler, 1978: Fig. 5).

As ontogenesis proceeds, the adoral shield spines show negative allometric growth. The infradental papillae change from a spinelike to a blocklike shape, and move from the dental plate to the tips of the oral plates by the time individuals reach 1.2 mm dd with 19 AS (Fig. 2D). In early stages the oral slits are tightly sealed by the series of teeth and oral papillae (infradental papillae and operculiform buccal scales) that are attached to the ventral edge of the jaw. As *Amphiura* grows, the surface of the jaw bulges ventrally, leaving the buccal scale attached deep within the oral slit. By the time the individual is 2.2 mm dd with 37 AS, enlargement of the proximal end of the scale transforms the buccal scale to a spine-shaped

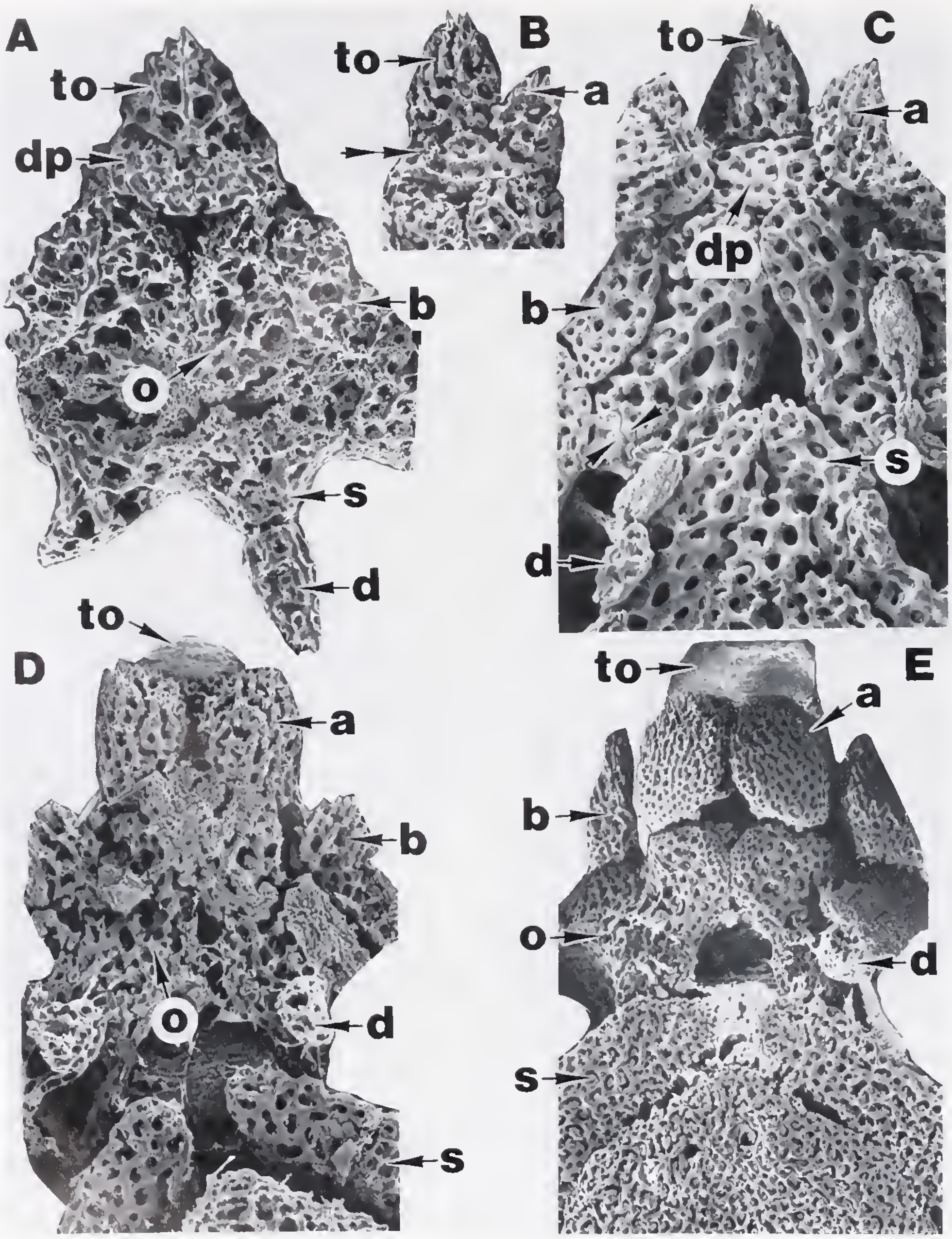

Figure 2. *Amphiura stimpsonii:* growth stages of jaw and oral papillae. Stages numbered according to Table 1. (A) Stage 2 (brooded); 0.51 mm dd, 4 AS. (B) Stage 4 (brooded); 0.9 mm dd, 10 AS; detail of jaw apex, double arrowhead indicates indentation on dental plate at attachment site for (missing) infradental papilla. (C) Stage 4 (free-living); 0.8 mm dd, 10 AS; opposing arrowheads indicate suture between proximal and distal oral plates of the jaw (*sensu* Hendler, 1978). (D) Stages 7–8; 1.2 mm dd, 19 AS; opposing arrowheads indicate suture between proximal and distal oral plates of the jaw (*sensu* Hendler, 1978). (E) Adult; 3.76 mm dd. Abbreviations: a, infradental papilla; b, buccal scale; d, adoral shield spine; dp, dental plate; o, oral plate; s, adoral shield; to, tooth.

oral papilla. In the adult (Fig. 2E), a gap separates the buccal scale from the first ventral arm plate and the oral region (initially sealed by the contiguous oral papillae) is incised by five large oral slits (Fig. 1B).

Throughout development the dorsal surface of the disk has an irregular arrangement of small scales (Fig. 1C). However, a distinct rosette with a central and five radial primary plates that is characteristic of many ophiuroids was never seen in *A. stimpsonii.* Five pairs of radial shields were found in a specimen 1.20 mm dd with 19 AS (also in a specimen with 15 AS, dd not measured). In individuals about 1.18 mm dd with 20 AS, the dorsal scales near the edge of the disk are markedly smaller than scales near the center, an indication that new scales originate at the periphery.

Amphiura filiformis *(O. F. Müller)*

The smallest juvenile, 0.30 mm dd, has arms with only a terminal plate; its oral frame resembles that of *Amphiura stimpsonii* of similar size. It already has two oral papillae, the buccal scales and adoral shield spines, that project beyond the periphery of the disc (Figs. 1D, 3A). The adoral shield spines are relatively larger than those in *A. stimpsonii* (Figs. 2A, 3A).

A. filiformis may have more arm segments than *A. stimpsonii* of approximately the same dd (Tables I, II). Oral skeleton ontogenesis progresses through the same sequence of stages as in *A. stimpsonii*. The infradental papillae, the third oral papillae to develop, form on the edge of the dental plate, become block-shaped, and eventually attach to the tip of the oral plate. The adoral shield spine migrates from the distal to the proximal end of the adoral shield, and then to the oral plate, growing to a relatively larger size than in *A. stimpsonii*. As *A. filiformis* grows, the enlargement of the jaws leaves the buccal scale attached deep in the oral slit. The buccal scale develops a sharp process at the proximal end, but the distal end (in contrast with the scale in *A. stimpsonii*) remains in contact with the first ventral arm plate. *Amphiura filiformis* develops a distal oral papilla ("accessory papilla" *sensu* Hendler, 1978) that is not found in *A. stimpsonii*. This papilla (not illustrated in Fig. 3) forms at the juncture of the adoral shield and first ventral arm plate as shown, for example, in Madsen (1970: Fig. 13).

The dorsal surface of the disk of newly settled *A. filiformis* has one central and five radial primary plates (Muus, 1981: Fig. 7), and radial shields are present in a specimen as small as 0.46 mm dd with 3 AS. The radial shields and primary plates are separated by a series of smaller disc scales in a specimen 0.84 mm dd with 25 AS; they are visible in large specimens and widely separated by numerous small disc scales of nearly uniform size.

Ophiophragmus filograneus *(Lyman)*

The smallest individuals studied, 0.50 mm dd with 1 AS, have buccal scales and adoral shield spines. A specimen 1.4 mm dd with 14 AS resembles *A. stimpsonii* at 0.9 to 1.2 mm dd with 10 to 20 AS. It has three oral papillae: the infradentals are spinelike, buccal scales are superficial, and adoral shield spines are still attached to the adoral shield. Two ontogenetic developments in *O. filograneus* radically depart from the pattern in *Amphiura* species. First, in specimens larger than 2.4 mm dd, all the buccal scales are resorbed (one exceptional specimen 2.5 mm dd with >35 AS still has buccal scales on one jaw). Second, a structure (the "oral plate papilla") which does not correspond to any of the oral papillae in *Amphiura* species arises between the base of the adoral shield and the infradental papilla (Fig. 4C, D; Tables I, II). Interestingly, another member of the *Amphiodia*-group, *Ophiocnida scabriuscula* resorbs the buccal scale—probably at the same growth stage that resorption occurs in *O. filograneus* (Tables I, II). A specimen 2.2 mm dd, with 61 AS, has vestiges of the buccal scales; adult individuals entirely lack the buccal scale (Hendler, unpub. obs.).

The adoral shield spine of *Ophiophragmus*, like that in *Amphiura*, migrates to the oral plate (Fig. 4B, C; Tables I, II). In large individuals the infradental papilla becomes blocklike, and the oral plate papilla and adoral shield spine become blunt and flattened. The oral papillae form a continuous series along the side of the jaw, but they do not seal the space between adjacent jaws.

The dorsal disc scales develop similarly in *O. filograneus* and *A. filiformis*. The smallest *O. filograneus* have a rosette of six primary plates, an individual 0.6 mm dd with 3 AS has radial shields, and the primary plates and radial shields are separated by small disc scales in an individual 1.4 mm dd with 14 AS. A fence of spines on the periphery of the disk, a distinctive character of *Ophiophragmus* species, is present in the 1.4 mm dd specimen and all the scales in the ventral interradii bear rough, pointed processes. These scales are smaller than but similar to the scales comprising the fence. In larger specimens, the fence spines increase in size and number, but large, smooth scales dominate the ventral interradii and surround a patch of rough scales. Thomas (1963) showed that adults and juvenile *Ophiophragmus cubanus* (H. L. Clark, 1917) were once treated as two species because of a similar contrast in the disc spination of large and small specimens.

Discussion

Conservative patterns of ontogenesis in amphiurids

Various amphiurid species have brooded embryos, yolky larvae with abbreviated development, or ophioplutei with different numbers of larval arms (Hendler, 1975). Considering the diversity in mode of development and the potential for modification of the developmental program, amphiurids would seem an unlikely group to examine for ontogenetic clues to systematic relationships. Interestingly, the patterns of oral papilla development shown in this study indicate that ontogenesis in postlarvae may be a reliable indicator of systematic relationships.

Species representing the major groups of Amphiuridae (*sensu* Clark, 1970), including taxa with different reproductive modes, all have related sequences of oral papilla development (Tables I, II). For example, the buccal scales and adoral shield spines develop before the infra-

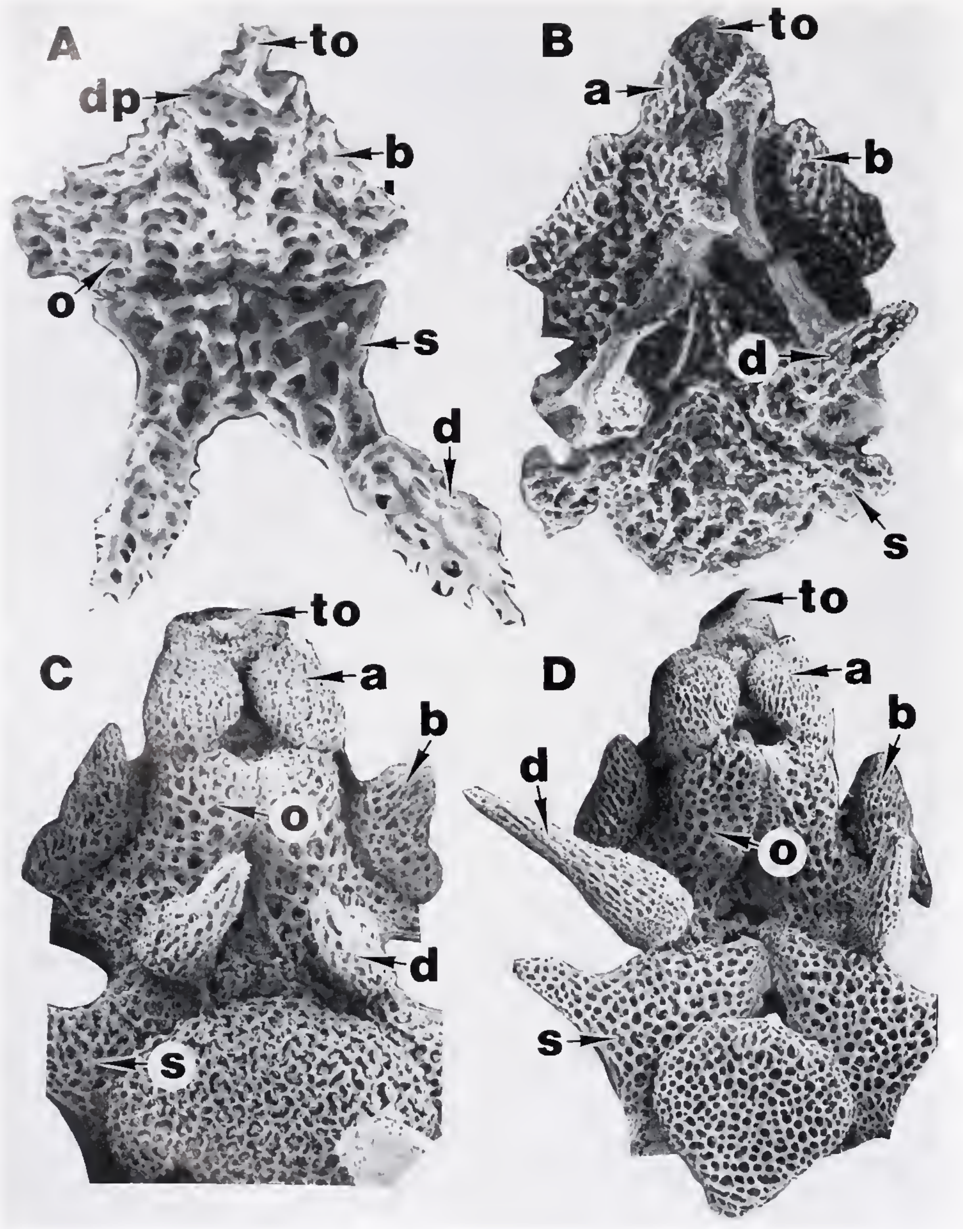

Figure 3. *Amphiura filiformis:* growth stages of jaw and oral papillae. Stages numbered according to Table I. Abbreviations as in Figure 2. (A) Stage 2; 0.33 mm dd, terminal arm plate. (B) Stage 4; 0.86 mm dd, 25 AS. (C) Stages 7–8; 3.20 mm dd. (D) Adult; 4.62 mm dd, >125 AS.

dental papillae in *A. filiformis* and *A. stimpsonii.* Late larval stages of *A. filiformis* have not yet been studied to determine whether the buccal scale appears before the adoral shield spine, as it does in *A. stimpsonii.* In both *Amphiura* species, the rudiments of infradental papillae originate on the dental plate and transfer to the tips of the oral plates; the buccal scales move from the superficial distal edge of the adoral shield to the oral plate. The intrageneric similarity in oral papilla ontogenesis of these two *Amphiura* species is paralleled by *Amphioplus macilentus* and *A. abditus,* congeners with nearly identical patterns of oral papilla ontogeny (Hendler, 1978; unpub. obs.; see Table I, this report). Similarities in the sequence of formation, origin, and morphological development of structures in related species provide a basis for determination of homologies of skeletal elements using standard morphological criteria of homology (Wiley, 1981).

Homologies of amphiurid oral papillae

Two aspects of the ontogenesis of *A. filiformis, A. stimpsonii,* and *O. filograneus* indicate that amphiurid

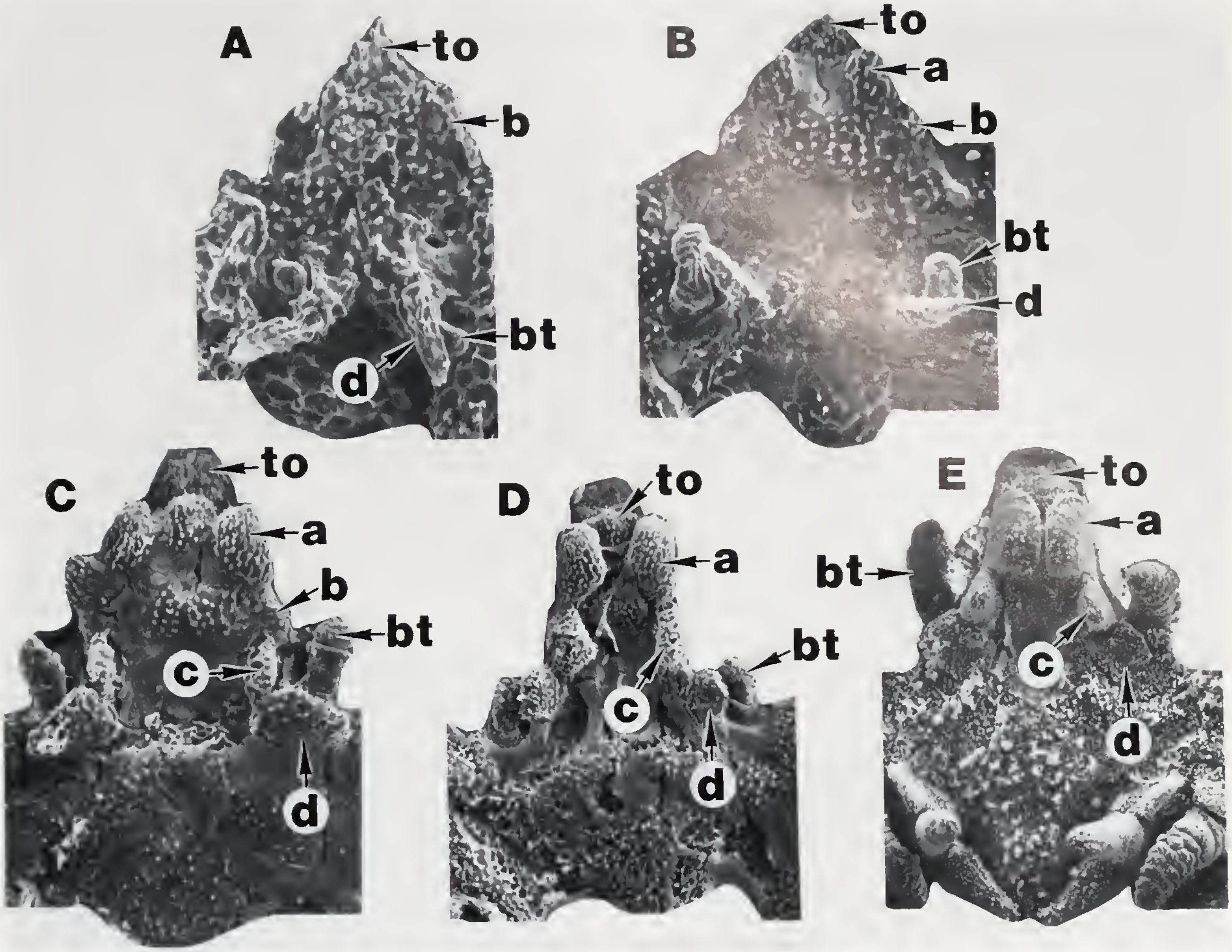

Figure 4. *Ophiophragmus filograneus:* growth stages of jaw and oral papillae. Stages numbered according to Figure 1; abbreviations as in Figure 2. The buccal tentacles (bt) are intact, and the oral plates and adoral shields are covered with integument because samples were not plasma-ashed to remove soft tissue. (A) Stage 2; 0.5 mm dd, 2 AS. (B) Stage 4; 1.4 mm dd, 14 AS. (C) Stages 5–8 initialized; 2.5 mm dd, >35 AS. (D) Stages 5–8 complete; 2.7 mm dd, >87 AS. (E) Adult; 6.9 mm dd, 319 AS.

oral papillae are not all derived from identical structures—that they are not serially homologous (*sensu* Roth, 1984). First, the oral papillae do not develop in a unidirectional sequence, such as proximal to distal along the jaw. Second, the anlage of each papilla is associated with a different skeletal plate. For example, as skeletal elements the adoral shields are regarded as homologues of the lateral arm plate, therefore the adoral shield spine (an oral papilla) is serially homologous with the arm spines that are attached to lateral arm plates of each arm segment (Hendler, 1978). In contrast, the association of the infradental papilla with the dental plate indicates that as a skeletal element it is homologous with dental papillae or teeth.

Specific oral papillae of different taxa are provisionally regarded as homologous if they originate at the same site and develop, or are resorbed, in the same chronological sequence. For example, in the taxa studied the buccal scale, adoral shield spine, infradental, oral plate papilla, and accessory papilla appear in sequence and each papilla forms at a characteristic site (Tables I, II). Thus, the buccal scales of all amphiurids are presumably homologous as oral papillae, the adoral shield spines of all amphiurids are presumably homologous as oral papillae, and so on.

Amphiurids lacking an "oral tentacle scale" (*sensu* Clark, 1970) resorb the buccal scale during ontogenesis; those with the "oral tentacle scale" retain the buccal scale. As previously predicted (Hendler, 1978), *Amphiura*-group and *Amphioplus*-group species retain the buccal scale, but *Amphipholis*-group and *Amphiodia*-group species resorb the buccal scale during ontogenesis (Table

I). It follows that the three oral papillae of *Amphiura stimpsonii* are not all homologous with those of *Amphipholis squamata* (compare the oral papillae of both species at stage 9 in Table I). In both taxa the infradental papillae are homologous, and the papillae derived from adoral shield spines are homologous. The middle papilla of *Amphiura* is derived from a buccal scale, but the buccal scale is resorbed in *Amphipholis* and its middle oral papilla (the oral plate papilla) arises late in ontogenesis and has no homologue in *Amphiura*. Furthermore, the four pairs of oral papillae in *Amphiura filiformis* are not all homologous with the first four pairs in *Amphioplus abditus* (Tables I, II). The four papillae in *Amphiura* (proximal to distal) are the infradental, buccal scale, adoral shield spine, and accessory papilla; the oral plate papilla (the third papilla in *Amphioplus* and *Ophiophragmus*) does not develop in *Amphiura* species.

In most cases, homologies have not been determined for the oral papillae that are important in the classification of ophiuroid families (Matsumoto, 1917). Developmental series could be used to evaluate such homologies. For that purpose, brooded embryos of representative species would likely be more readily available than growth series of free-living young. It is important that the ontogenesis of the oral papillae of brooded embryos and free-living juveniles discussed in this report are so very similar since this is an indication that homologies inferred from growth series of brooded embryos can be applied to species with free-living larvae. Thus, homologies inferred from character transformation series in brooded embryos can be used to assess the systematic affinities of species with planktonic larvae. Information on ontogenetic character transformations derived from growth series is also applicable to the identification of young individuals in ecological studies. In samples from the deep-sea, for example, where juvenile ophiuroids are disproportionately abundant, they are so morphologically different from adults that they often cannot be identified even to family or genus (Grassle and Sanders, 1973; pers. obs.).

Phylogenetic relationships suggested between taxa are strictly tentative if they are derived solely from oral papilla homologies that are based on ontogenetic patterns. Wherever possible, systematic relationships inferred from ontogenesis should be independently corroborated (see Alberch, 1985). However, ignoring the relationships suggested by ontogenesis would be "throwing out the baby with the bathwater."

Skeletal adaptations of juvenile amphiurids

The adaptive significance and variability of skeletal features such as oral papillae bear upon their usefulness for interpreting systematic and evolutionary relationships. Several observations suggest that the oral papillae, despite their changing functional roles during ontogenesis, are consistent and reliable taxonomic characters.

At 1 year, the mean dd of *Amphiura filiformis* is 0.6 mm (range 0.4–0.8) (Muus, 1981). *Amphioplus abditus* reaches a similar size (0.4–0.8 mm dd) by 8 months (Hendler, 1978). Their oral papillae still show the juvenile arrangement of small spine-like infradentals, opercular buccal scales, and distally directed adoral shield spines. Not until 1 to 2 years after metamorphosis does the oral armature assume adult characteristics. The brooded embryos of *A. stimpsonii* hatch at about the same size and with the same oral armature as the year-old, free-living juveniles that are on the verge of transformation to adult morphology.

Although the radical changes of the oral armature which distinguish the juvenile and adult occur over a long period, the earliest post-metamorphic stages of *A. filiformis* can ingest food items large enough to fill the entire disk (Muus, 1981). The functions of all the juvenile oral structures are not yet known but surprisingly, free-living juveniles probably use the adoral shield spines for locomotion and not for feeding. The adoral shield spine in the brooded embryo of *Amphiura stimpsonii* is very small and possibly vestigial, but free-living post-larvae of *A. filiformis, O. filograneus,* and *Amphioplus abditus* (Figs. 1A, D, 2A, 3A, 4A this report; Hendler, 1978) have relatively much longer adoral shield spines Until the 8 AS stage, *A. abditus* juveniles walk on the tips of their tube feet (Hendler, 1977), and the youngest stages of *A. abditus* walk on the second buccal papillae and use the adoral shield spines to support the disc during locomotion (Hendler, unpub. obs.). Unfortunately, the function of the adoral shield spine in advanced growth stages and the significance of interspecific differences in its morphology in adults have not been investigated.

Interestingly, the adoral shield spine is present in the juveniles of bottom-dwelling ophiuroids, and absent in epizoic taxa such as Asteronychidae and Ophiotrichidae (examples in Mortensen, 1912; Guille, 1964). However, juveniles of the latter taxa have large hooked arm spines, probably used for clinging and crawling, which show marked negative allometric growth. The specialized arm spines and the presence or absence of the adoral shield spine in the young may be adaptations for locomotion on the preferred substratum. Thus, there may be gross differences between the oral papillae of ophiuroid families with radically different ecological specializations. However, within a family such as the Amphiuridae the oral papillae are conservative features, even among species with different developmental modes.

Some commonly used taxonomic characters were found to be less consistent among related species. For example, a rosette consisting of one central and five radial plates is present on the disc of *A. filiformis* and absent in *A. stimpsonii*. The difference in scalation may be related to their modes of reproduction, but the absence of a rosette is not characteristic of all brooding species. For example, viviparous *Amphipholis squamata* develops from 0.10 mm eggs (Fell, 1946), and has a primary rosette (Murakami, 1940). In contrast, *Sigsbeia conifera* Koehler, a brooding species with relatively yolky eggs, lacks a primary rosette (Hendler and Littman, 1986; Hendler, unpub. obs.). Mortensen (1936) reports brooding species with (*e.g., Ophiozonella falklandica* Mortensen) and without (*e.g., Ophiomages cristatus* Koehler) primary rosettes. I am not aware of any ophiuroid species with planktonic larvae that do not develop a primary rosette, but in some brooding forms absence of the rosette is congenital. The presence or absence of a central rosette is clearly of limited value for deducing genealogical relationships among ophiuroids. However, investigations of disk scalation might make it possible to infer the mode of reproduction from the adult morphology of extant as well as fossil ophiuroids.

Acknowledgments

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H_2S—A Settlement Cue or a Toxic Substance For *Capitella* sp. I Larvae?

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Abstract. In small-scale laboratory experiments, organic-rich sediment lacking sulphide elicited settlement and metamorphosis in freshly hatched *Capitella* sp. I larvae, so that 90% settled within 30 min after hatching. Settlement times of somewhat older larvae were even shorter; 90% settled into the mud in less than 5 min. The addition of sulphide to these treatments (0.5 m*M*, 1.0 m*M*, and 2.0 m*M*) delayed settlement, so that it took several hours for 90% of the larvae to settle. Many of these larvae showed abnormal behavior and settled distant from the sediment. Sulphide alone (without sediment) enhanced settlement in a concentration-dependent manner (0.5–2.0 m*M*), as previously reported by Cuomo (1985). However, this response occurred over 12–24 h and abnormal larval settling behavior was observed. Hypoxia produced a similar response.

Considerations of behavior and swimming capabilities of *Capitella* larvae, near-bottom hydrodynamic conditions in the field, and the time course of these responses to organic-rich sediment, sulphide, and hypoxia, lead to the conclusion that sulphide is not a settlement cue promoting habitat selection in *Capitella* sp. I larvae. The apparent enhancement of settlement by sulphide is hypothesized to be a sub-lethal toxic effect.

Introduction

The opportunistic polychaete, *Capitella capitata*, is regularly found in sediments of high organic content (Pearson and Rosenberg, 1978), where bacterial decomposition can lead to high concentrations of H_2S. Cuomo (1985) described H_2S as a settlement cue for *Capitella* sp. I (as determined by Grassle and Grassle, 1976) larvae. This was the first time that H_2S was shown to induce a larval response, and Cuomo hypothesized that this cue might explain the high abundances of *Capitella* species in sulphide-rich sediments.

However, there may be an alternative explanation for the effects of H_2S on the settlement of *Capitella* sp. I larvae. The toxicity of H_2S is well documented (National Research Council, 1979) but the effects are reversible when concentrations diminish (Degn and Kristensen, 1981; Torrans and Clemens, 1982). Sub-lethal concentrations of H_2S could trigger larval settlement and/or metamorphosis by physiologically 'shocking' the larvae. Then, as concentrations diminish, recovery might occur with subsequent normal development. Since H_2S is toxic to most living organisms, it would probably 'shock' other larvae besides *Capitella* thereby enhancing settlement of many different species. Other explanations for the high abundances of *Capitella* in H_2S-rich sediments, *e.g.*, differences in survival of larvae and adults, would seem more likely.

To test the proposed 'toxicity' hypothesis, *in vitro* experiments similar to those of Cuomo (1985) were performed. *Capitella* sp. I larvae were exposed to different H_2S concentrations in petri dishes for 24 h. Care was taken to observe behavior throughout the entire settlement process. Additional experiments were conducted to investigate (a) behavior in the presence of a natural substrate by introducing sediment and then testing settlement time with and without H_2S, (b) how long larvae take to settle after hatching by observing settlement behavior of freshly hatched larvae, and (c) the specificity of the settlement response to H_2S by inhibiting the same metabolic pathways as H_2S, namely with hypoxia.

Materials and Methods

In this paper the term "settlement" is defined as termination of pelagic larval existence and denotes a behavioral response (Scheltema, 1974). "Metamorphosis" is a

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morphological term and describes a developmental process (Burke, 1983). In experiments with sediment, settlement time was noted when larvae entered the mud. In experiments without sediment, settlement as defined above did not occur. To compare results, I designated cessation of effective swimming after loss of prototrochal and telotrochal cilia as settlement. However, as shown in the Discussion, I argue that this process is better defined as metamorphosis.

Capitella sp. I larvae were used in all experiments. This species is one of the most opportunistic of the sibling species complex described by Grassle and Grassle (1976) (previously determined as one single species *Capitella capitata* Fabricius). Larvae were obtained from mass cultures reared at ambient seawater temperatures in the laboratory of Dr. J. P. Grassle (Marine Biological Laboratory, Woods Hole). Worms with brood tubes containing developing embryos were isolated in individual dishes without sediment at 20°C and checked daily for hatching of larvae. All experiments were done at room temperature (21–23°C) in 55 mm diameter glass petri dishes.

Experimental sediment consisted of azoic, organic-rich mud from Sippewissett marsh (MA) that had been passed through a 1 mm mesh sieve, frozen, thawed, and aerated twice. Adult worms were cultured in similar sediment but the mud used in experiments was freshly thawed and had not been used for cultures. Sulphide was not detected in the mud with the colorimetric technique used (minimum detectable conc.: 70 μM) or identifiable by odor (perception threshold: approx. 0.1 μM; Dando *et al.*, 1985).

Settlement behavior of freshly hatched larvae

Larvae were tested for settlement ability immediately after hatching. Brood tubes were examined daily until hatching seemed imminent. Early embryos, visible through the wall of the brood tube, are opaque and filled with whitish yolk, and lack any distinct morphological features. Through the 5–6 days of development at 20°C they gradually change, gaining the features of the fully developed metatrochophore larva. The yolk disappears and the gut takes on a greenish blue cast. Shortly before hatching they start to move within the tube. From this point on, the tube was watched continually until larvae began hatching. All the larvae in a brood tube did not hatch simultaneously; hatching took up to an hour with sometimes one larva, and sometimes several larvae at once crawling and swimming out of the brood tube. This behavior made it impossible to record individual settlement times and therefore brood tubes were opened manually, causing all larvae to hatch simultaneously.

As soon as the first 'naturally' hatching larvae were observed, the brood tube was quickly placed in a dish filled with 30 ml of 0.2 μM filtered seawater. Experimental azoic sediment was placed in a small clump in the middle of the dish and the tube torn apart with two dissecting needles. Behavior was examined with a dissecting microscope and two fiber-optic ring lights placed at opposite sides of the dish until all larvae had settled.

H_2S and hypoxia experiments

The behavior and settlement of *Capitella* sp. I larvae were observed under the conditions listed in Table I. Experiments were designed to investigate sub-lethal toxic effects of H_2S. Since Cuomo (1985) found 10 mM sulphide to be toxic to most *Capitella* sp. I larvae, concentrations between 0.1 and 2.0 mM sulphide were tested. In the field, sulphide concentrations can vary greatly depending on the measurement method used, the type of sediment, the time of year, the depth at which the sample was taken, etc. In the same New England salt marsh sulphide concentrations in the sediment ranged from as low as 1.2 μM (Howarth *et al.*, 1983) to as high as 6 mM (Howes *et al.*, 1985).

Most experiments were run in parallel, *i.e.*, each treatment was tested at least once on a given day with sibling larvae from a single brood. Time was a limiting factor because each experiment required at least one hour of constant observation before the next could be started. Larvae were no older than 24 h post-hatching at the beginning of each experimental day. Since up to nine different treatments were tested on one brood, larvae used at the end of the day were, in some cases, at least 9 h older than those used in the morning. Thus, care was taken to change the sequence in which treatments were tested.

H_2S experiments: H_2S and pH determinations. All sulphide measurements were made colorimetrically using a modified version of the Gilboa-Garber method (Howarth *et al.*, 1983). The desired sulphide concentrations were achieved by adding the needed amount of 100 mM sulphide stock solution. Stock solutions were made by dissolving $Na_2S \cdot 9H_2O$ crystals in deoxygenated, deionized water.

The sulphide concentrations measured just before the larvae were added are shown in Table II. The decreases in sulphide after 1 h, measured in some experiments, are shown in Table III.

All pH measurements were made with an electrode. Deoxygenation through N_2 bubbling and addition of H_2S caused high pH values (up to 9.5) in the dishes. The pH was always adjusted to that of untreated seawater (approx. 8.0) by adding 2.0% HCl. Actual pH values varied between 7.6 and 8.1; the average pH in all experiments (n = 89) was 7.97 ± 0.13.

H_2S experiments: H_2S without sediment. Thirty ml of deoxygenated, 0.2 μm filtered seawater were poured into

Table I

Experimental conditions

	H_2S									Hypoxia	
Sulphide	With sediment				Without sediment						
conc. (m*M*)	0*	0.5	1.0	2.0	0*	0.1	0.5	1.0	2.0	$+O_2$ *	$-O_2$
# of replicates	30	7	16	6	7	4	6	7	6	6	6

* = Controls.

a petri dish and the needed amount of 100 m*M* sulphide stock solution added to reach the concentrations listed in Table I. The pH was measured and adjusted to that of untreated seawater. After measuring the sulphide concentration, five larvae were pipetted into the petri dish and the dish was covered with a watch glass. Only larvae from the same brood were used in each experiment. Behavior and settlement were observed continually throughout the first hour using a dissecting microscope with two fiber-optic ring lights on opposite sides of the petri dish as a light source. Dishes were examined every 15 min for the following 2 h and every 30 min thereafter for a total of 24 h. Controls were treated as above, except that no H_2S was added.

H_2S experiments: H_2S with sediment. At the beginning of each experiment a small clump of experimental sediment was placed in the middle of a petri dish. Thirty ml of deoxygenated, 0.2 μm filtered seawater were added carefully so that the mud remained in the center. The same procedure was followed as described above (under *H_2S without sediment*). Larval settlement and behavior were observed continuously for the first hour, every 5 min for the second hour, and every 10 min thereafter until all larvae had settled.

Hypoxia experiments. The effect of reduced oxygen concentrations on larval settlement behavior was tested under conditions similar to the H_2S treatments without sediment. Five or ten larvae (all from one brood) were pipetted into a dish which was then carefully filled to the brim (approx. 35 ml) with 0.2 μm filtered seawater that had been bubbled with N_2 for at least 30 min. The dish was quickly covered with a watch glass of the same size, convex side down, so that there were no bubbles. The rim of the dish was wrapped with adhesive tape. Behavior and settlement were observed for the same time periods and under the same conditions as the H_2S experiments. Controls were treated in the same manner but dishes were not wrapped with tape.

Results

Settlement behavior of freshly hatched larvae

Settlement time and behavior were similar in all seven broods observed. Fifty percent of all larvae tested settled into the sediment in less than 5.5 min (Fig. 1) and in 5 broods the remaining 50% settled within 13–17 min. In two broods settlement times of approx. 30% of the larvae were somewhat longer with the last larvae taking up to 45–55 min to settle.

The decrease in settlement with time in Figure 1 is due to the fact that as the larvae settled, fewer were left in the pelagic stage. Therefore, settlement only appears to fall off. Settlement can be shown to be constant (*i.e.*, not drop off with time) in this treatment by plotting the function log (N/N − x) (where x is the number settled and N is the total number originally present in the dish; Crisp, 1974). (I preferred to present all data as % settled to emphasize the actual number of larvae settled at any given time rather than the rate of settlement with time.)

Table II

Average sulphide concentrations (measured just before each experiment began)

Treatment	Measured sulphide conc. in m*M* ($\bar{x} \pm s$)	n (# of experiments)
0.1 m*M*	0.098 ± 0.010	4
0.5 m*M*	0.508 ± 0.051	13
1.0 m*M*	1.030 ± 0.045	23
2.0 m*M*	2.040 ± 0.109	12

Table III

Decrease in sulphide after 1 h in some experiments

Treatment	Decrease in sulphide in % ($\bar{x} \pm s$)	n (# of experiments)
0.1 m*M*	17.7 ± 2.3	2
0.5 m*M*	12.9 ± 3.0	5
1.0 m*M*	14.6 ± 4.5	11
2.0 m*M*	10.2 ± 2.0	3

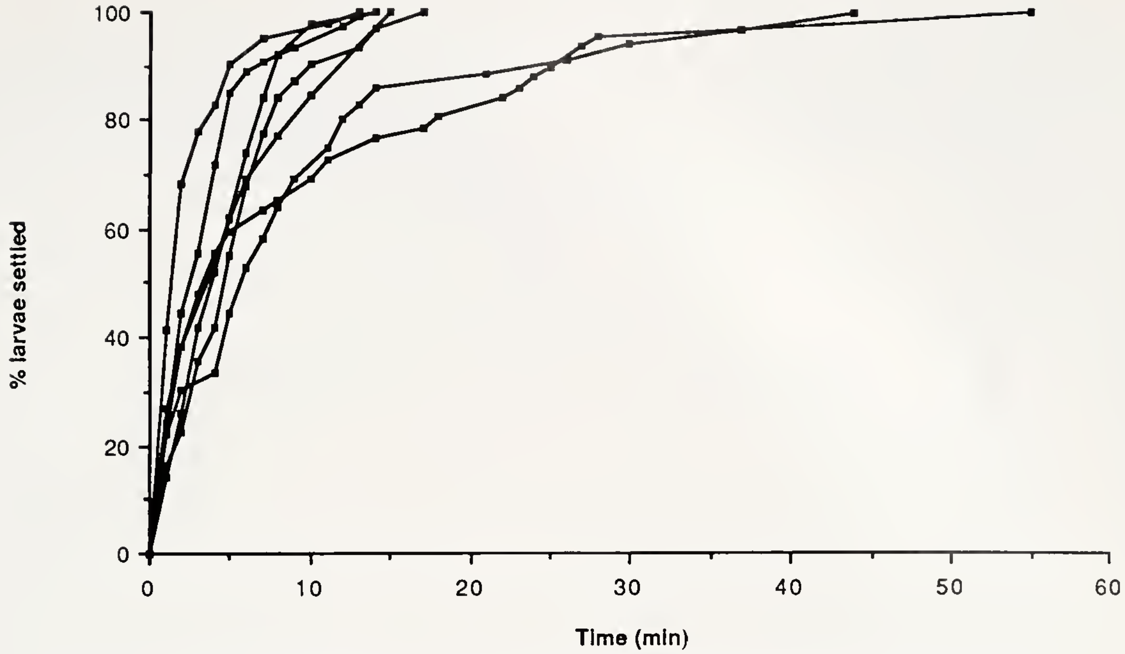


Figure 1. Settlement of freshly hatched *Capitella* sp. I larvae in sediment. Each curve represents settlement times of sibling larvae from one brood; 7 broods with between 13 and 74 larvae were tested. Settlement was observed continuously until all larvae settled.

Behavior. For these experiments the brood tubes were opened manually when the larvae began hatching (see Materials and Methods). However, there was no difference in behavior between these and 'naturally' hatched larvae. All larvae showed positive phototaxis, as described for this species by Butman *et al.* (in press). Upon leaving the brood tube, larvae immediately swam up to the surface and then toward the light sources on opposite sides of the petri dish. Larvae that settled within minutes then turned away from the light, swam down towards the mud, and crawled into it (at which point settlement time was recorded). Larvae that took longer to settle regularly swam back and forth between both light sources or clumped at the brighter light source (one ring light was inevitably a little closer than the other to the dish) before swimming down to and entering the mud. Only rarely would larvae test the mud and swim away again; larvae almost never left the mud after settling. On some occasions loss of cilia was observed within the next 5–10 min.

In several instances where brood tubes were not torn apart, I observed a few larvae that metamorphosed within the brood tube over a period of 1–2 h. The juvenile worms then left the tube and crawled into the mud.

H_2S experiments with sediment

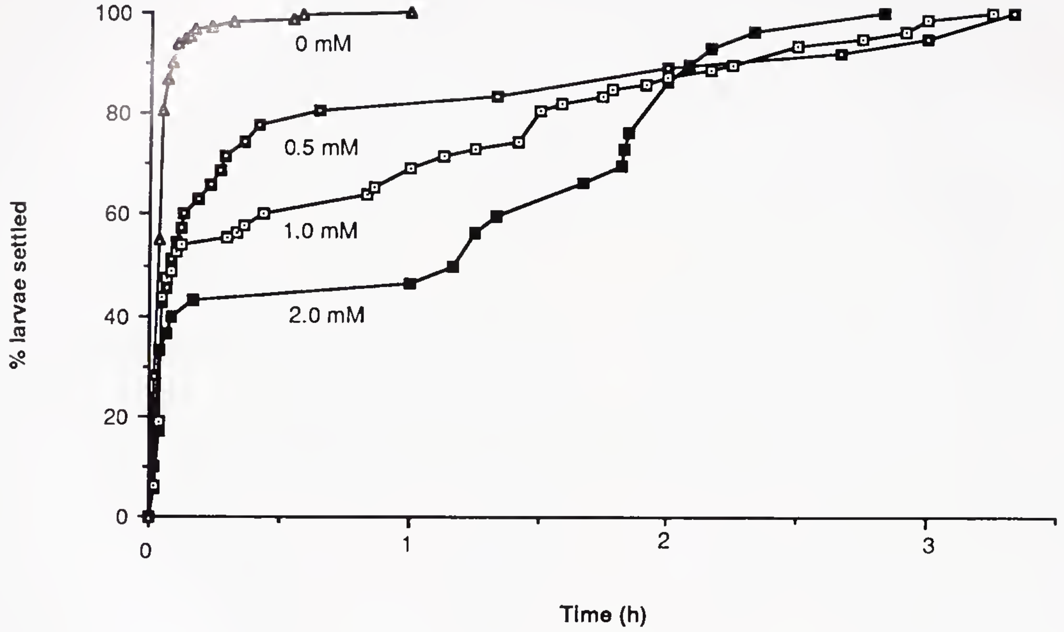
In the presence of sediment alone behavior of older larvae (up to 35 h post-hatching) was similar to that of freshly hatched larvae, while settlement was even quicker. Fifty percent settled within 1 min, 90% within 5 min, and only 3 of 150 larvae tested took longer than 30 min to settle (Fig. 2a, Table IV a). In the presence of H_2S much longer settlement times were recorded. With increasing sulphide concentrations a higher percentage of larvae delayed settlement ($\bar{x}$ in last column in Table IV a).

Logarithmic transformation of the data for Figure 2a showed that the decrease in settlement with time in all three sulphide treatments is genuine, while settlement in the absence of H_2S does not drop off with time.

Behavior. In all treatments larvae always swam toward the light sources after introduction to the dish. In the absence of sulphide behavior was as described for freshly hatched larvae. In the presence of H_2S behavior was different depending on how quickly the larvae settled. If they settled within minutes they behaved like the freshly hatched larvae, quickly entering the mud. Some larvae that took longer to settle would repeatedly swim toward the mud, touch it with their prostomium, turn around, and swim away again (usually toward the light) until they eventually crawled into the mud; others behaved like those in the H_2S experiments without sediment and settled on the bottom of the dish away from the sediment.

H_2S and hypoxia experiments without sediment

Little or no settlement occurred in the H_2S and hypoxia controls and at the lowest (0.1 m*M*) sulphide concentration (Fig. 2b, Table IV b). Enhanced larval settlement was recorded in the 0.5, 1.0, and 2.0 m*M* sulphide


a) with sediment

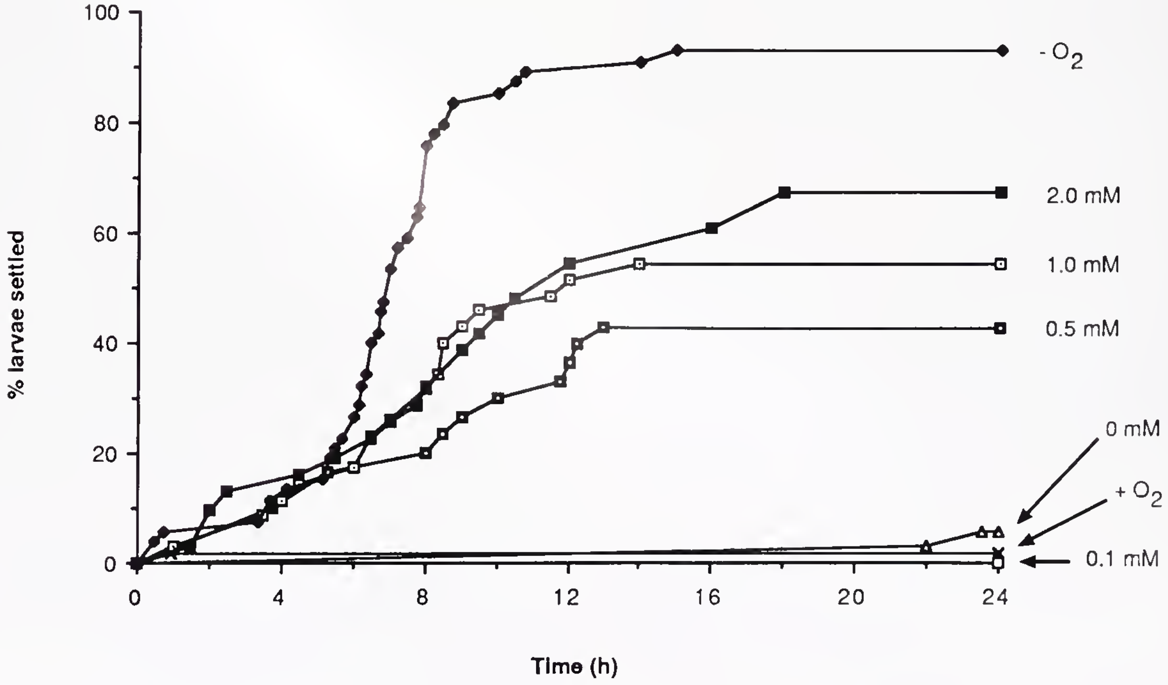

b) without sediment

Figure 2. Settlement of *Capitella* sp. I larvae in various H_2S concentrations and hypoxia. (a) With sediment (note time scale of 3 h). Number of larvae tested: 30 (2.0 m*M*); 35 (0.5 m*M*); 80 (1.0 m*M*); 150 (0 m*M*). (b) Without sediment (note time scale of 24 h). Number of larvae tested: 20 (0.1 m*M*); 30 (0.5 and 2.0 m*M*); 35 (0 and 1.0 m*M*); 55 ($-O_2$ and $+O_2$). Each curve represents pooled data from all experiments at one treatment level. $+O_2$ = control for hypoxia treatments ($-O_2$); 0 m*M* = control for sulphide treatments (0.1, 0.5, 1.0, and 2.0 m*M*).

Table IV

Percent settlement of Capitella *sp. I larvae in various H_2S concentrations and hypoxia. (a) Percent settlement after 15 min in experiments with sediment; (b) percent settlement after 24 h in experiments without sediment. (Columns represent different treatments tested within one day on sibling larvae from one brood. Rows represent one treatment tested on different days with different broods.)*

		BROOD																			
	Treatment	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	$\bar{x} \pm s$
With sediment	(a)																				
	0 m*M*[1]	100	100	100	100	100	100	80	100	100	80	100	100	100	100	100					
							100	60			100				100						96 ± 10
								100			100										
	0.5 m*M*						0				80	100	60	40	100	80					66 ± 36
	1.0 m*M*	20	80	60	80	40	0	40		40	0	100	100	40	100	20					54 ± 34
										80		60									
	2.0 m*M*								0			40	100	0	100	20					43 ± 46
Without sediment	(b)																				
	0 m*M*[1]									0	0	0	0	0	40	0					6 ± 15
	0.1 m*M*												0	0	0	0					0 ± 0
	0.5 m*M*										100	60	0	40	60	0					43 ± 39
	1.0 m*M*									100	100	80	0	80	20	0					54 ± 46
	2.0 m*M*											80	40	60	100	100					67 ± 33
																20					
	$+O_2$[2]														0*	0	0*	0*	0*	10*	2 ± 4
	$-O_2$														100*	40	100*	100*	100*	100*	90 ± 24

* In these experiments 10 larvae, instead of 5 as in all other experiments, were used.

[1] Control for H_2S treatments.

[2] Control for hypoxia treatments.

treatments with 43, 55, and 68%, respectively, of all larvae tested settling within 24 h. However, settlement times in all three sulphide concentrations were much longer than in treatments with sediment, with less than 20% of the larvae settling within the first 6 h and less than 50% after 10 h. Higher settlement and the shortest settlement times in the absence of sediment were observed in the hypoxia experiments where 50% settled within 7 h and 90% settled within 11 h. The decrease in settlement with time (in Fig. 2b) in the three highest sulphide and hypoxia treatments is genuine.

Behavior. As in all other treatments, larvae showed a more or less pronounced phototaxis by swimming back and forth between the two light sources or clumping toward the brighter light source.

A gradual elongation of the body was visible in all larvae that eventually settled. (I was not able to distinguish if this was due to a gain in segments.) As they grew longer the larvae swam more slowly and spent more time on the bottom of the dish. They would still regularly swim up from the bottom of the dish as long as they had cilia. After the telotrochal cilia were lost, the larvae would still occasionally lift their 'heads' up from the bottom but could no longer swim. Once the prototrochal cilia were lost they remained flat on the bottom. This point was defined as settlement.

Larvae that did not settle also appeared to increase in length, but over a much longer time period (>12 h). Swimming speed decreased but not as quickly as in larvae that eventually settled.

Variation in settlement times within and among broods

Table IV shows variation in settlement times among and within broods. Low within brood variation means that either all or none of the larvae settled, as represented by 100% and 0% in the table. Among brood variation is represented by s. More than one value for a brood in a treatment means that this treatment was replicated.

In all control experiments and at the lowest sulphide concentration (0.1 m*M*) there was almost no variation in settlement times within and among broods. However, at all higher H_2S concentrations settlement time varied greatly in both cases. In comparison, the response to hypoxia was much more uniform where, with the exception of one brood, all tested larvae settled within 24 h.

Discussion

The experiments with freshly hatched larvae showed that more than 40% of the larvae tested settled almost immediately after hatching and virtually all larvae settled into the sediment in less than an hour. These results show that *Capitella* sp. I larvae are competent to respond to a settlement cue within minutes after hatching.

Somewhat older larvae (up to 35 h post-hatching) were used in all other experiments. In the presence of sediment alone, settlement behavior of older larvae did not differ from that of freshly hatched larvae. Settlement times were even shorter with 50% settling within 1 min and 96% in less than 15 min. This might be expected since it is well known that with increasing age the settlement 'drive' becomes stronger in some other species of larvae (Crisp, 1974).

When 0.5 m*M*, 1.0 m*M*, and 2.0 m*M* sulphide was added to the sediment, settlement times increased up to several hours. In the absence of sediment, sulphide concentrations greater than 0.1 m*M* enhanced larval settlement, but it took up to 12 h for less than 50% of the larvae to settle.

These results question the role of H_2S as a settlement cue for *Capitella* sp. I larvae. Mud, without detectable H_2S, elicits settlement within minutes, whereas the settlement response to H_2S occurs over a time span of many hours.

The time course of Cuomo's (1985) experiments was 8 days in the settlement choice, 2 months in the microcosm, and 24 h in the *in vitro* and optimal concentration experiments. In the settlement choice and microcosm experiments sediment was used but direct observation of larval settlement was not possible. Cuomo noted that the experiments did not allow "a distinction to be made between sulphide as a potential energy source (bacterial growth as food) for juvenile *Capitella* sp. I and as a settlement cue for the larvae" (p. 174). Cuomo therefore designed the 24 h *in vitro* and optimal concentration experiments in petri dishes to better isolate and investigate the effects of sulphide. However, she did not use sediment in these experiments and thus could not observe the quick settlement response of *Capitella* sp. I larvae to mud.

The enhanced larval settlement under hypoxia and in the presence of H_2S in the experiments without sediment could be a sub-lethal toxic effect. Cuomo found that an initial concentration of 10 m*M* sulphide was lethal to most *Capitella* sp. I larvae, but at initial concentrations of 1.0 m*M* sulphide, larvae metamorphosed successfully and went on to develop and grow normally at a lower sulphide concentration (0.1 m*M* range). In this study no acute toxic effects such as arrest of ciliary movement were observed at initial concentrations up to 2.0 m*M* sulphide. However, the following factors support the toxicity hypothesis and call into question the role of sulphide as a settlement cue:

(1) The experiments without sediment by themselves might lead to the conclusion that H_2S is a settlement cue, but the larvae would be expected to show the strongest settlement response to mud plus H_2S. Instead, settlement times were prolonged up to several hours. This would be contradictory if H_2S were a settlement cue, but can be explained if the tested sulphide concentrations were sublethally toxic. With increasing sulphide concentrations a higher percentage of larvae were adversely affected and prevented from settling quickly into the mud. The behavior of larvae that settled on the bottom of the dishes away from the mud supports this hypothesis.

(2) If active habitat selection occurs in soft-bottom animals, one would expect larvae to choose an environment suitable for life. "Mechanisms must exist to ensure that larvae are brought into contact with the correct substratum, thus ensuring heaviest settlement in the most favoured substratum and least settlement in unsuitable substrata" (Gray, 1974). The strongest settlement response in the no sediment experiments was to hypoxia, but in treatments continued for up to 48 h most settled larvae died (results not shown). A similar 'response' occurred at 10 m*M* sulphide in Cuomo's experiments where highest settlement was recorded but most settled larvae died within 24 h.

One could argue that lower H_2S and higher O_2 concentrations would not be lethal. One might then expect a strong response to a lower, optimal H_2S concentration. However, in both this study and Cuomo's study, settlement *decreased* with diminishing H_2S concentrations, *i.e.*, there was no evidence for an H_2S optimum.

(3) In the sediment experiments without H_2S all larvae showed a rapid, uniform response (Table IV a) as would be expected for a settlement cue. The broad variation in the response to H_2S, not only between broods but also among siblings from the same brood (Table IV a), would mean that in the field approx. 50% of the population would not settle quickly (within minutes) in the presence of the tested sulphide concentrations. Hydrodynamic considerations (Butman, 1986) make a quick response necessary, however, and make it very unlikely that H_2S can be a cue. The broad variation in behavior is much more reminiscent of that observed in many toxicity tests, with an increasing proportion of animals adversely affected by H_2S as concentrations rise, taking longer to settle into the sediment, or failing to locate it at all.

Although care was taken to keep experimental pH fluctuations small, the standard deviation of pH values was 0.13. Since the undissolved H_2S molecule is thought to be more toxic than HS^- (National Research Council, 1979; Powell and Somero, 1986) and the degree of dissociation is mainly pH-dependent (Millero, 1986), the observed settlement variations could have been caused by differences in the concentrations of undissolved H_2S. However, correlation between various concentrations of undissolved H_2S and settlement percentages could not be proven ($R = -0.11$) in the treatment with the highest range of pH variation (1.0 m*M* sulphide with sediment: pH 7.6–8.1). Here, undissolved H_2S contents varied between 3 and 9% of the total sulphide concentration; apparently these variations were too small to affect settlement behavior.

(4) The distinction between settlement and metamorphosis is not always clear and was not defined by Cuomo (1985). Settlement in the sense of Scheltema (1974) denotes a responsive behavior and refers to the termination of pelagic existence. Metamorphosis on the other hand,

is a morphological term and is characterized by substantial morphological and physiological changes. In experiments with sediment, settlement occurred when larvae swam into the mud and stayed there; metamorphosis, defined as loss of cilia and elongation by addition of setigers (Butman *et al.*, in press), followed shortly afterwards. In experiments without sediment a behavioral response was never observed. The larvae in a slow 'metamorphosis' gradually lost their cilia, sank to the bottom of the dish, and stayed there. Metamorphosis caused loss of swimming ability—settlement as defined above never occurred.

Abnormal metamorphosis in response to sub-lethal doses of toxic substances (*e.g.*, Cu salts, ethanol, vital dyes) has been described by a number of authors (reviewed in Crisp, 1974). Although the processes involved in this abnormal metamorphosis are unclear, Crisp proposed that these substances act directly on and modify the metabolism of the larvae. H_2S, through its high affinity for cytochrome-c-oxidase (Evans, 1967; Smith *et al.*, 1977) and lack of oxygen inhibit the same metabolic pathway: aerobic respiration. The slow, abnormal metamorphosis of larvae in the H_2S and hypoxia experiments without sediment seems indicative of a modification of the larvae's metabolism whereas the quick settlement and metamorphosis in the presence of organic-rich mud is much closer to a behavioral model of stimulus and response (Burke, 1983).

Regardless of whether H_2S is considered to be a settlement cue or as here indicated sub-lethally toxic—the protracted response to H_2S over a time span of many hours is unlikely to be important for settlement in sulphide-rich sediments in the field. Although *Capitella* sp. I larvae are competent to respond to a settlement cue within minutes after hatching, their behavior (immediately swimming up away from the brood tube at hatching and toward the light) makes it likely that most larvae will spend a certain amount of time in the plankton. There, the following settlement scenario as proposed by Butman *et al.* (in press) can be suggested: selection of sites is usually not possible by horizontal swimming because water flow speeds within several mm of the seabed generally exceed swim speeds of *Capitella* sp. I larvae by factors of two to an order of magnitude (Butman, 1986; Butman *et al.*, in press). This means that while larvae can actively reject unsuitable upstream habitats, and test (and/or accept) a downstream location through vertical swimming, selection of sites is hydrodynamically constrained. Substrate-testing behavior of *Capitella* sp. I larvae can occur almost immediately after hatching, is quick, and is followed by abrupt swimming away if the substrate is rejected. Therefore the protracted response to H_2S, where hours could pass before settlement ensued, should be of no importance in active habitat selection especially when compared with a cue that elicits settlement within minutes.

The high abundances of *Capitella* in sulphide-rich sediments can not be explained by an active response of the larvae to H_2S. Since such sediments are also high in organic content, larval response to one or several cues in organic-rich mud could influence settlement at these sites. In experiments by Chesney (1985) and Chesney and Tenore (1985), the quantity of administered food (Gerber mixed cereal) significantly affected settlement rates of *Capitella* sp. I larvae, and the authors proposed that the stimulus for settlement was a "chemical cue associated with organic matter."

For effective settlement of sulphide-rich sediments, concentrations in the water column would have to be lower than 0.5 mM sulphide, as abnormal settlement behavior and protracted settlement times were observed at sulphide concentrations ≥ 0.5 mM. These conditions would exist over any sulphidic sediments covered by oxic water, since H_2S is oxidized quickly and the H_2S-O_2 interface is usually very narrow (Jorgensen, 1982). In the two long-term settlement experiments conducted by Cuomo (1985), exactly such conditions existed (sulphide ranged between 0.01 mM and 0.1 mM).

Thus organic-rich sediment appears to be a strong settlement cue for *Capitella* sp. I larvae. Certainly further research is needed to elucidate the effects of various physical, biological, and chemical components of organic-rich mud on active habitat selection.

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Distributional Consequences of Adhesive Eggs and Anural Development in the Ascidian *Molgula pacifica* (Huntsman, 1912)

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Abstract. *Molgula pacifica* (Huntsman) is a recently rediscovered ascidian that occupies shallow subtidal rocks on wave-swept coasts of British Columbia. Individuals occur most abundantly at sites with intermediate exposure at or near 4 m depth. On a scale of centimeters, they are highly aggregated. *Molgula pacifica* is hermaphroditic, self-fertile, and oviparous. Embryos develop on the bottom without passing through a typical tadpole stage. Each of the egg follicle cells contains a single large adhesive vacuole that occupies most of the cell volume. Shortly after spawning these vacuoles rupture, causing the follicle cells to secrete a sticky mucus coat that adheres the egg to the substratum. Juveniles hatch and move away from the chorion using epidermal ampullae, as reported for other anural molgulids. Adhesive eggs may be an adaptation that permits anural development in high-energy hard-bottom habitats. Egg adhesion may also explain the small-scale distribution of the species.

Introduction

> ". . . it is very probable that (anural development) is to be correlated with the outstanding peculiarity of the family, namely, its unattached sand-flat habitat and its adaptation to such an existence." (Berrill, 1931)

Most ascidians pass through a "urodele" or tadpole larval stage. Suppression of the tailed tadpole (termed "anural development") is known only in the families Molgulidae (Lacaze-Duthiers, 1874; Damas, 1902; Berrill, 1931) and Styelidae (Millar, 1954, 1962), though within the Subphylum Urochordata, tailed larvae are also lacking in the development of most thaliaceans (Berrill, 1950). Anural development has been previously observed in 10 molgulid species (Berrill, 1931), eight of which live on sandy or muddy bottoms. Only two urodele species, *Molgula oculata* and *M. occidentalis,* inhabit soft bottoms. Conversely, on hard substratum, 13 of the 15 molgulid species with known developmental modes demonstrate normal urodele development (Berrill, 1931; Whittaker, 1979; Torrence and Cloney, 1981). Both known anural styelids, *Pelenaia corrugata* (Millar, 1954) and *Polycarpa tinctor* (Millar, 1962), live on sandy substrata. Berrill (1931) reasoned that urodele development is an ancestral condition that gave rise to anural development among sand-dwelling species because larval swimming and habitat selection have little value where the substratum is flat and homogeneous. By extension of this argument, he suggested that the few attached anural species represent reinvasions by sand-dwelling forms of the ancestral hard-bottom habitat (Berrill, 1931). Whittaker's (1979) discovery of vestigial tail muscle acetylcholinesterase in anural species provides indirect support for these ideas. Although we might expect that anural species colonizing rocky substrata would somehow compensate for their inability to swim, no compensatory features have been described.

Huntsman (1912) described *Molgula pacifica* from a single specimen collected at Ucluelet, British Columbia. Besides a redescription by Van Name (1945) based on this same specimen, nothing more is known about the biology of this species. We recently discovered a large subtidal population of *Molgula pacifica* in Barkley Sound, British Columbia, approximately 35 km from the

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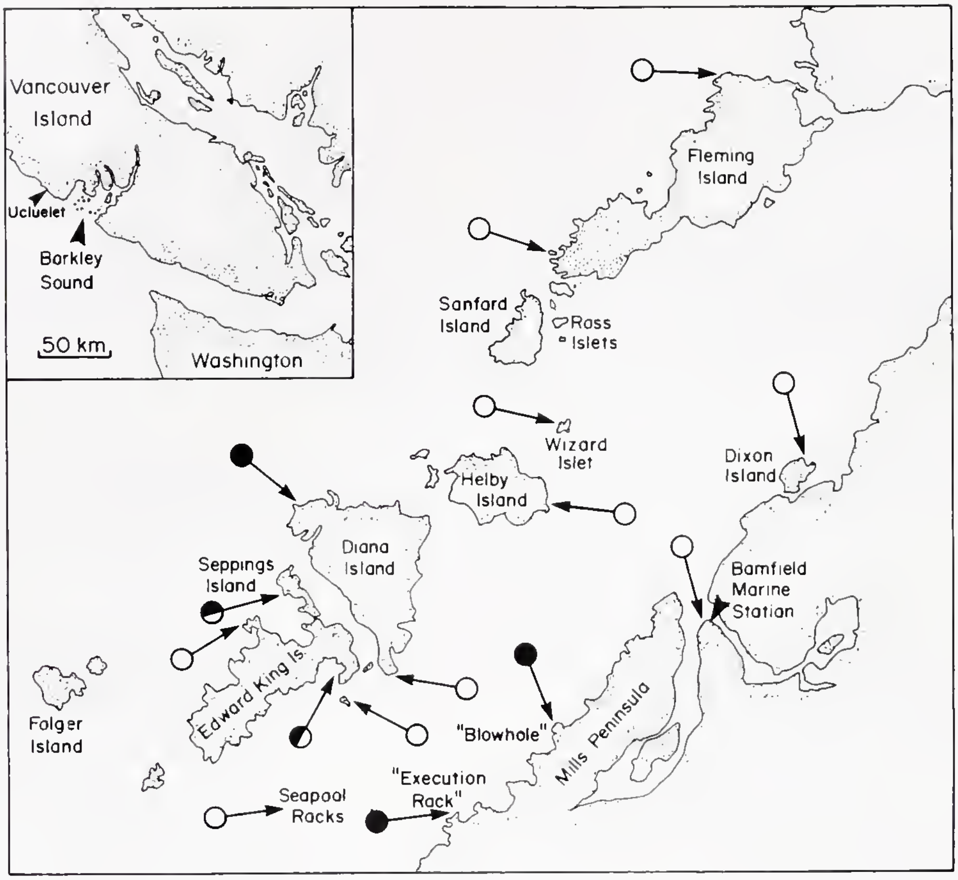

Vancouver Island
Ucluelet
Borkley Sound
50 km
Washington
Fleming Island
Sanford Island
Ross Islets
Wizard Islet
Dixon Island
Helby Island
Diana Island
Seppings Island
Bamfield Marine Station
Edward King Is.
Folger Island
"Blowhole"
Mills Peninsula
"Execution Rock"
Seapool Rocks

Figure 1. Map of the study region on the southeastern edge of Barkley Sound, British Columbia, Canada. Arrows indicate subtidal sites that were systematically surveyed for *Molgula pacifica* populations. Circles attached to the tails of the arrows give qualitative density values as follows: closed circle: abundant; open circle: absent; half-shaded circle: present in low numbers.

type locality. In this paper, we describe the general characteristics of this species' habitat, give a quantitative analysis of distributional patterns on several scales, present a general description of its anural development, and report an unusual developmental adaptation that may permit exploitation of high-energy subtidal habitats. A redescription of the species will be presented elsewhere (Pennechetti *et al.*, in prep.).

Materials and Methods

Distributional surveys

We studied the distribution of *Molgula pacifica* at three scales. Large-scale qualitative surveys were made at 16 sites along a 10 km exposure gradient in Barkley Sound, British Columbia. At each site, at least 4 divers began at 10 m depth and worked up the slope, stopping to search more carefully at 6 m, 4 m, and 2 m depths. No quantitative data were taken on these survey dives. Field notes consisted of observations on sizes of individuals, animal and plant associates, depth of occurrence, surface angles of occupied rocks, and wave surge.

At an intermediate scale, quantitative data were taken at the Blowhole site on the southern shore of Barkley Sound, where *Molgula pacifica* occurred abundantly. Within each of two long surge channels (called Blowhole sites 1 and 2 hereafter), we counted all individuals in five randomly positioned 50 × 50 cm quadrats at each of three depths (6 m, 4 m, 2 m). Within each depth, three habitat types were surveyed: (1) gentle slopes or horizontal surfaces in channel bottoms, (2) vertical surfaces on the sides of channels, and (3) horizontal or sloping ridges or plateaus between surge channels. The data for each site were analyzed by 2-way ANOVA in which both factors (depth and habitat) were fixed.

Small-scale (within-habitat) distribution was quantified from underwater photographs taken of the rock surface. At each of 4 sites (3 at Blowhole, 1 at Execution Rock), we photographed a 6 × 6 grid of contiguous rectangular quadrats on rock walls or steep slopes. Each quadrat encompassed an area 15 × 22 cm. Transparencies were projected and counted by at least three individuals before the data were compared with expected random (poisson) distributions using goodness of fit tests.

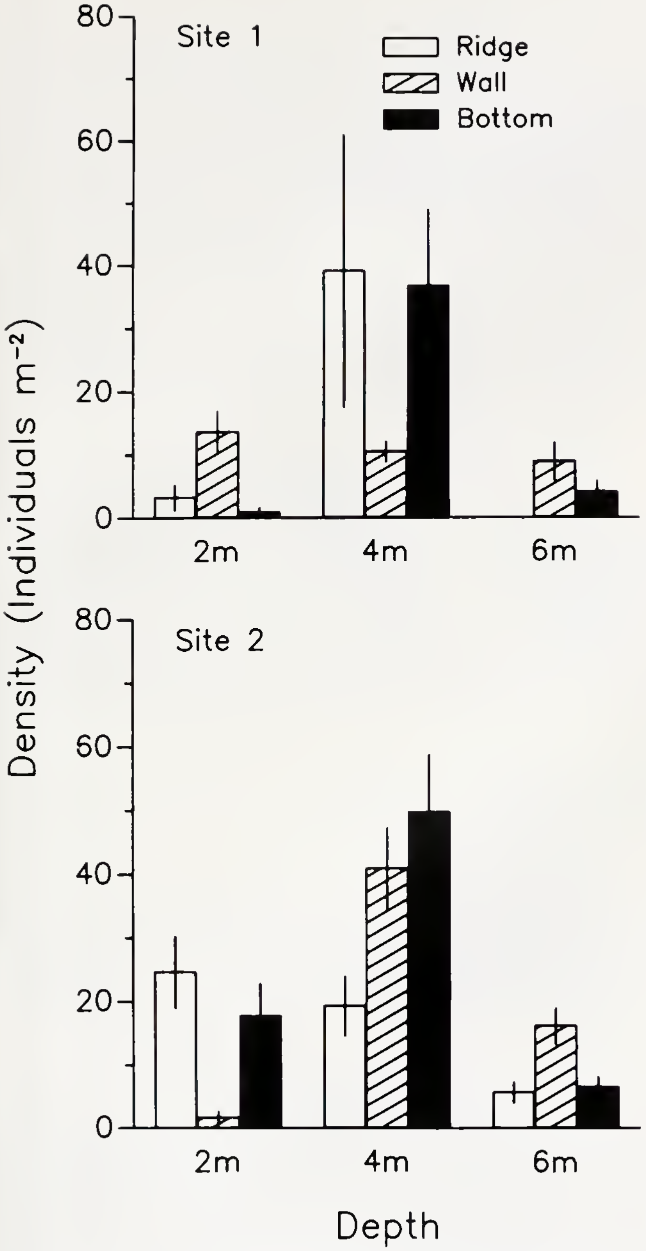


Figure 2. Densities of *Molgula pacifica* at three depth/habitat combinations in two surge channel systems at the Blowhole site. Error bars represent one standard deviation on each side of the mean. Data analysis is presented in Table 1.

Other species of solitary ascidians were also counted in one grid (Blowhole grid 1) to determine if their densities correlated with those of *Molgula pacifica.*

Embryology and histology

Individuals of *Molgula pacifica* were collected by scuba from the Blowhole site in Trevor Channel, British Columbia, during July 1985, and placed in seawater tables at the Bamfield Marine Station within one hour of collection. Gonads were removed from some adults by dissection, then macerated through 253 μm nitex monofilament mesh. Other individuals released gametes spontaneously in seawater tables. Following fertilization, excess sperm were removed by repeated rinsing with fresh seawater. Cultures were maintained at 10°–12°C in a shallow, flow-through seawater table. We attempted to induce spawning by light shock by incubating eight individuals in darkness for 12.0 h, then exposing them to the subdued light of the laboratory.

Follicle cells of dissected eggs did not secrete adhesive spontaneously. We induced holocrine secretion for histological study by placing the eggs in hypotonic seawater (1 part seawater, 2 parts distilled water). Eggs treated in this way appeared identical to eggs spawned naturally in the laboratory.

Embryos were fixed in Torrence's fixative for 2.5 h, followed by three 20-minute rinses in Torrence's buffer (Torrence and Cloney, 1981). Embryos were fixed, rinsed, and dehydrated on ice, then brought to room temperature during the first change of absolute isopropanol. They were embedded in Luft's Epon 812 (Luft, 1961) and sectioned on a Reichert OMU2 ultramicrotome using glass knives. Thin sections were stained with uranyl acetate, post-stained with lead citrate, and examined at 60 kV using a Phillips EM 300 electron microscope. To aid in the recognition of mucus, 1-μm sections were stained with an aqueous solution of 0.25% toluidine blue in 0.5% sodium borate (Humason, 1967; R. Burke, pers. comm.).

Living embryos and juveniles were observed and photographed on Leitz or Zeiss photomicroscopes using phase contrast, bright field, or Nomarski optics.

Results

Distribution

The distribution of *Molgula pacifica* showed clear patterns at three scales. Figure 1 shows the large-scale distribution, as determined by qualitative surveys, within the southern portion of Barkley Sound and the Deer Island group. The mouth of the sound experiences the rough surf and surge conditions of the open Pacific coast. Wave action decreases regularly along the southern shore of the sound because of protection from the Deer Islands and Broken Islands. The conditions at any given site in the Deer group depend on the distance of the site from the mouth of the sound, protection by other islands, and direction of exposure. Individuals of *M. pacifica* were not found at either the most exposed sites or the most protected sites. The sites at which they were most abundant had moderate exposures with constant, year-round surge. This pattern is apparent along at least three exposure gradients (Fig. 1). Along the southeastern shore of Barkley Sound abundances went from zero at Seapool rocks to high density at Execution Rock and Blowhole,

Table I

Analyses of variance testing the importance of habitat (channel wall, channel bottom, ridge between channels) and depth (2,4,6 m) on densities of Molgula pacifica *in two channel systems at the Blowhole site (Fig. 2)*

Source of variation	d.f.	SS	MS	F	*P*
Site 1:					
Depth	2	2.763	1.381	23.55	0.000
Habitat	2	0.260	0.130	2.22	0.123
Depth × habitat	4	1.485	0.371	6.33	0.001
Error	36	2.112	0.058	—	—
Site 2:					
Depth	2	2.048	1.024	29.03	0.000
Habitat	2	0.092	0.046	1.30	0.284
Depth × habitat	4	2.089	0.522	14.81	0.000
Error	36	1.270	0.035	—	—

then dropped to zero at Bamfield Inlet and Dixon Island. On the leeward side of the Deer group, where the exposure gradient is more abrupt, intermediate numbers of animals were found at Edward King Island, but none were found on the southern tip of Diana Island or on the Eastern Shore of Helby Island. Finally, on the windward side of the Deer group, no animals were found on an exposed northern tip of Edward King Island, intermediate numbers were present on Seppings Island, high numbers were found on Diana Island, and abundances dropped to zero on the northwestern sides of Wizard Islet and Fleming Island.

The two parallel surge channels surveyed at the Blowhole site (Fig. 2) had different overall densities of *Molgula pacifica,* so the distributions were analyzed with separate two-way analyses of variance (Table I). The same general pattern was apparent at both sites. The main factor of depth and the depth × habitat interaction (where habitat refers to channel bottoms, sides, or the ridges between channels) were significant in both cases. At site 1, about 6 times as many *M. pacifica* occurred at 4 m depth than at either 2 m or 6 m. Qualitatively, the same pattern was seen at site 2. The interaction at site 1 resulted from most individuals occurring on the channel walls at 2 m and 6 m, but more occurring on channel bottoms and ridges at 4 m (Fig. 2). The distributions are similar between sites at 6 m, but differed substantially at 2 m and 4 m. At site 2, very few individuals occurred on channel walls at 2 m. At 4 m, approximately equal numbers occurred on the walls and channel bottoms (Fig. 2).

Frequency distributions of the abundance data from the small-scale photographic grids were compared with poisson distributions to determine the nature and significance of small-scale clumping (Fig. 3). With the exception of Blowhole grid 1, which had a much lower overall density than the other three, all of the observed distributions differed significantly from expected random distributions. Numerous quadrats with zero values and quadrats on the upper tails of the observed frequency distributions (densities as high as 727.2 animals per m^2 in individual quadrats) indicate aggregated spatial distributions. To visualize the nature of the clumps, we depict the raw data as three-dimensional plots (Fig. 4). Each grid encompassed a rectangular area 1.188 m^2. Clumps were generally on the scale of several quadrats (*i.e.,* tens of centimeters). At Execution Rock, on a gently sloping rock face, there was a sharp density gradient that went from a region with few animals in one corner to a single large aggregation that occupied most of the remainder of the plot.

To determine if the aggregations of *Molgula pacifica* occupied the same sites as aggregations of ascidians with tailed tadpoles, we computed correlation coefficients in which *M. pacifica* abundances were paired with abundances of three other species occurring at Blowhole grid 1. Two of the species, *Pyura haustor* and *Chelyosoma productum,* are strongly gregarious as larvae, whereas the third, *Cnemidocarpa finmarkiensis,* settles randomly

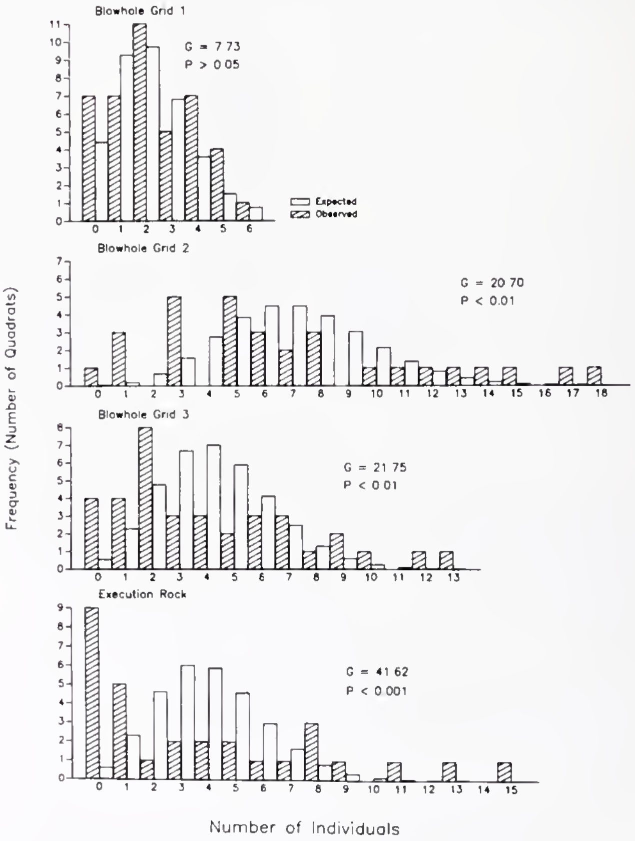


Figure 3. Observed (shaded bars) and poisson (open bars) frequency distributions of *Molgula pacifica* in small-scale photographic grids. In computing the goodness of fit (G) values for each site, classes with expected frequencies less than 2.5 were combined on the tails to make the significance tests more conservative.

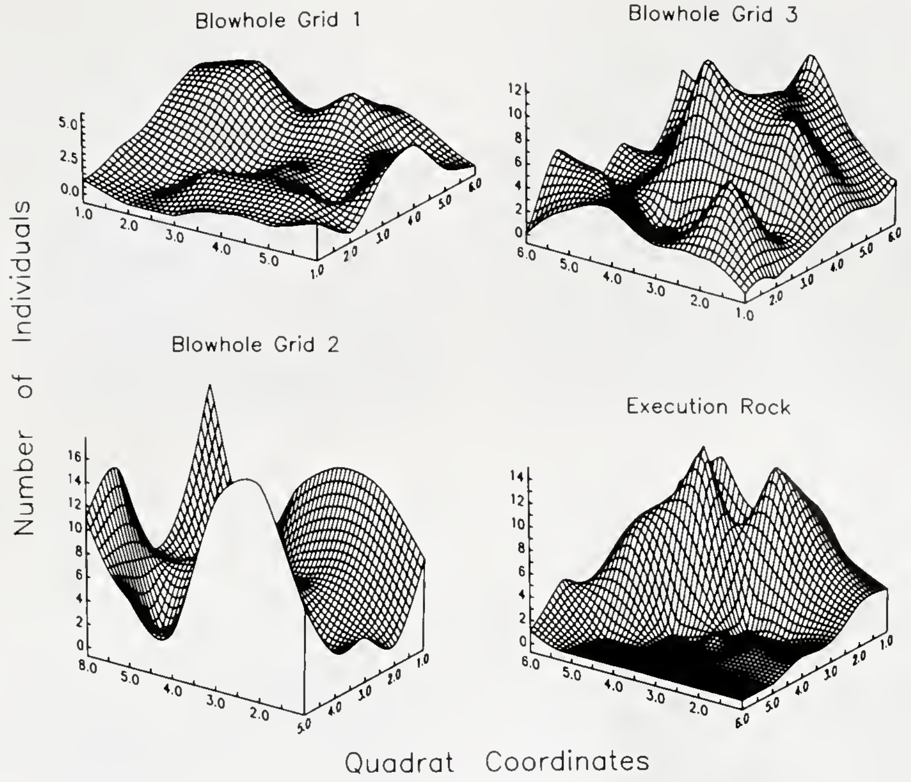


Figure 4. Small-scale abundance patterns of *Molgula pacifica* at four sites.

with respect to established individuals (Young, 1982; Young and Braithwaite, 1980). None of the correlation coefficients were significant (*P. haustor:* r = 0.005; *C. productum:* r = −0.002; *C. finmarkiensis:* r = −0.244; P > 0.05 for all three).

Reproduction and development

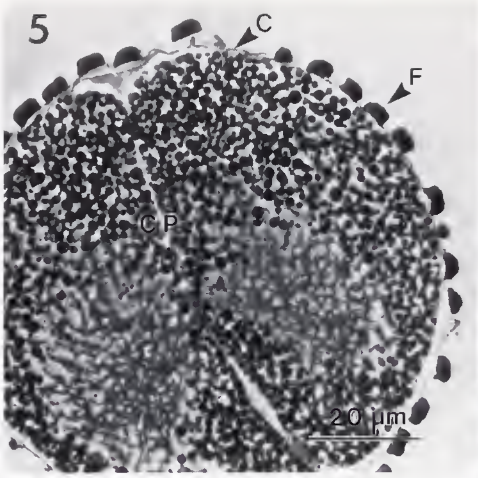
Unlike many ascidians that spawn during the day following dark adaptation, *Molgula pacifica* released gametes during periods of darkness. All eight individuals tested spawned, and all released gametes during, not after, the dark adaptation period. Approximately 100–200 eggs were released by each individual. Adults were maintained in separate dishes during these experiments, yet all embryos were undergoing development when they were discovered in the morning. In dissecting numerous specimens we never found embryos being brooded within the atrial chamber. Thus, *M. pacifica* is an oviparous hermaphrodite capable of self fertilization.

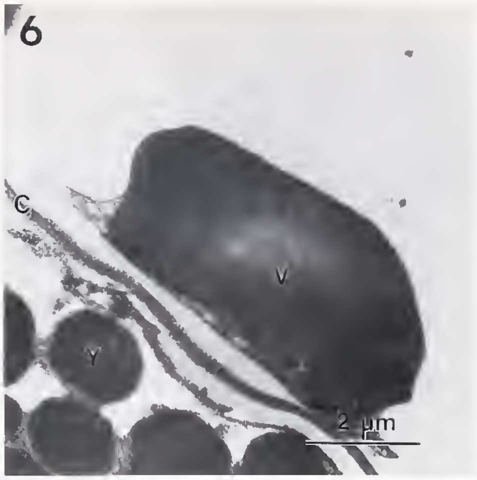
The eggs are 180 μm in diameter, including the follicle cells. At spawning, the egg has a single outer layer of follicle cells surrounding the chorion (Fig. 5). The chorion is closely applied to the egg plasma membrane; thus, the perivitelline space is much smaller than in most typical oviparous ascidians (Fig. 6). Sections of the eggs revealed no test cells (Fig. 5, 6), but careful observation of living material showed a few test cells present (R. Cloney, pers. comm.). Each follicle cell contains a single large vacuole which occupies most of the cell volume. Shortly after spawning, these vacuoles undergo a holocrine secretion of their contents to form the adhesive coat (Fig. 7, 8). This coat, which stains intensely with toluidine blue, swells to occupy a space many times the volume of the original follicle cell (Fig. 9). Those portions of the follicle cell membranes not disrupted by the secretory process remain closely applied to the chorion after secretion (Fig. 8), but all traces of the follicle cells disappear by 24 h after fertilization.

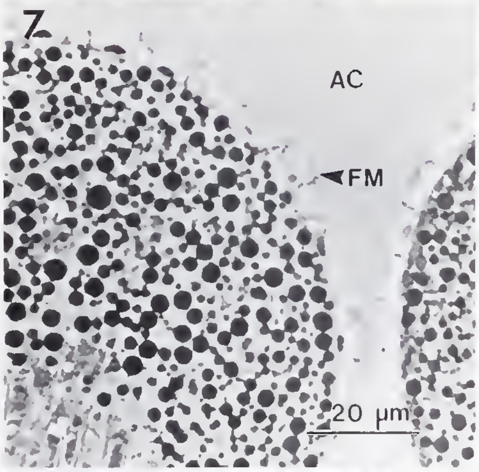
All but a few of the naturally spawned eggs were firmly attached to the substratum at the time they were discovered. Many of these were clumped together and joined by a common adhesive coat (Fig. 9).

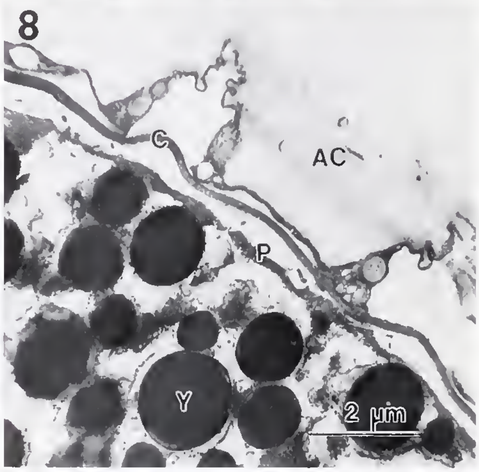
Eggs removed from the ovaries by dissection did not produce adhesive coats spontaneously. Nevertheless, many were sticky and their follicular vacuoles could be induced to release their contents by exposure to a hypotonic medium. Following secretion, such eggs appeared identical to naturally spawned eggs with adhesive coats.

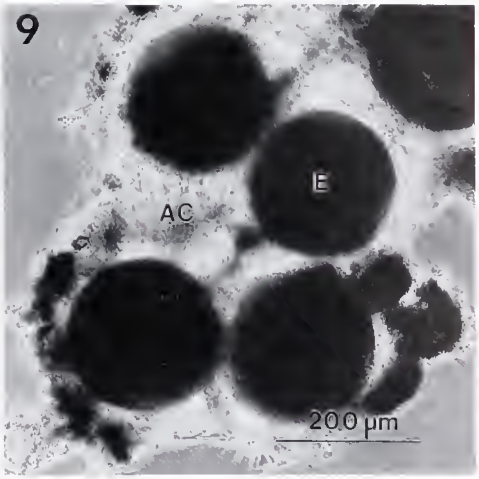
Complete embryonic development occurs within the adhesive coat; no tailed tapole larva is ever produced. The early details of anural development in this species are obscured by the opacity of the adhesive coat and embryo. At 10–12°C, the first juveniles hatched 36 h after











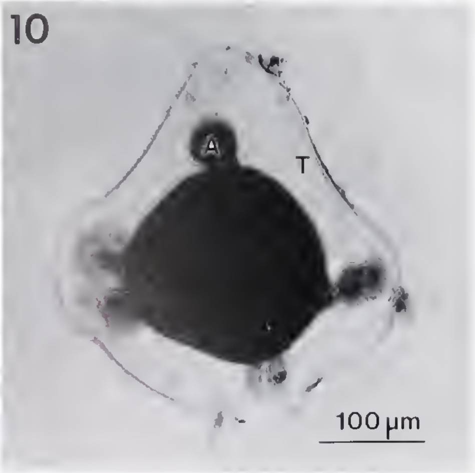


Figure 5. One-micron section of naturally spawned 2-cell embryo just prior to secretion of the adhesive coat. Note the very thin perivitelline space and apparent absence of test cells. Stained with toluidine blue. F: follicle cell. C.P.: cleavage plane. C: chorion.

Figure 6. Electron micrograph of a follicle cell prior to secretion of adhesive coat. V: vacuole. C: chorion. Y: yolk granule.

Figure 7. Thick (1-μm) section of an egg following secretion of the adhesive coat. AC: adhesive coat. FM: follicle cell membranes.

Figure 8. Electron micrograph of an artificially removed egg just after secretion of adhesive has been induced by exposure to hypotonic seawater. C: chorion. P: plasmalemma. AC: Adhesive coat. Y: yolk granule.

Figure 9. Aggregation of naturally spawned eggs (E) bound together by common adhesive coat (AC).

Figure 10. Juvenile shortly after hatching. A: ampulla. T: tunic.

fertilization. Just before hatching, a variable number (1–5) of epidermal ampullae were extended onto the substratum (Fig. 10). As in other molgulids, embryos hatch by rupture of the chorion. One ampulla was often larger than the others, and juveniles generally extended this ampulla through the initial fissure in the chorion. No embryos were observed to hatch before any ampullae had formed, but some hatched with only a single ampulla. The broken adhesive coats remained attached to the substratum for several days after hatching, then deteriorated.

Discussion

Molgula pacifica is one of only three molgulids known to have anural development while occupying hard substratum, and it is the only known anural species in the Pacific Ocean. It is particularly surprising that the species lives in wave-swept habitats. We propose that the adhesive eggs may be an adaptation that permits this unexpected habitat distribution, as the eggs probably stick to surfaces near the parents and develop on the bottom. Tadpoles of other species living in this habitat presumably use their swimming ability to assist in reaching the substratum.

The highly clumped small-scale distribution may be explained by two mechanisms associated with egg adhesion. First, eggs frequently attach to each other after spawning; indeed, they often share a common adhesive coat. Such egg masses falling to the bottom would place numerous individuals in close proximity. Although ampullar locomotion probably disperses individuals away from the initial attachment site at hatching, such locomotion would not be expected to obliterate the clumped pattern. Aggregations produced in this manner by siblings should be dominated by single size classes. Second, local aggregations could be established and maintained by limited (philopatric) dispersal. It seems reasonable to assume that eggs should be more likely to contact the substratum near their parents than further away. These juveniles in turn would produce short-distance dispersing offspring of their own, resulting in aggregations with polymodal size distributions. We were unable to make precise size measurements of individuals in our photographs because of adjacent epifauna. Nevertheless, it is clear from the photos that clumps tend to contain animals of all sizes, suggesting that the second method of aggregation could be important.

Philopatric dispersal is known in many other sessile organisms (reviewed by Jackson, 1986), including some that occupy high-energy habitats. For example, the intertidal alga *Postelsia palmeformis*, which lives only on exposed headlands, often forms aggregations containing individuals of all sizes (Dayton, 1973). During low tides, spores are released from sori located in grooves of the drooping blades. The spores flow down the grooves and fall on or near the haptera of the parents, where they adhere (Dayton, 1973).

Some ascidians with active tadpole larvae settle gregariously, whereas others settle randomly (Young, 1982; Young and Braithwaite, 1980). Both kinds of species occurred in the same habitats as *Molgula pacifica*. *Chelyosoma productum*, which aggregates behaviorally *in vitro* (Young and Braithwaite, 1980) and in the field (Young, 1982) formed clumps at about the same scale as those of *M. pacifica* at our study sites. However, the densities of the two species were not correlated, suggesting that the aggregations are formed and maintained independently. *M. pacifica* densities were also not correlated with those of *Pyura haustor*, another gregarious species, nor with *Cnemidocarpa finmarkiensis*, a species that does not aggregate. If some sites were consistently better for juvenile survival than others, or if current patterns concentrated propagules in certain regions, we would expect positive correlations among ascidian species since neither of these processes should discriminate among tadpoles of different species. The low correlation coefficients do not support the idea that ascidians accumulate passively. Thus, clumps of *Chelyosoma productum* and *Pyura haustor* are probably established by tadpole behavior, whereas similar clumps of *M. pacifica* are probably formed by philopatric dispersal mediated by sticky eggs.

In the egg of a typical urodele ascidian, test cells are located within superficial concavities of the oocyte (Kessel and Kemp, 1962). A single layer of inner follicle cells is separated from the oocyte and test cells by the chorion. Prior to ovulation, a layer of outer follicle cells surrounds the inner follicle cell layer (Kessel, 1983). At ovulation, the outer follicle cell layer remains in the ovary with the germinal epithelium, the chorion lifts away from the plasma membrane of the oocyte, and the test cells are extruded from their superficial concavities and come to lie within the perivitelline space. The eggs of some anural ascidians (*e.g.*, *Molgula bleizi*) contain test cells and a perivitelline space, whereas the eggs of others (*e.g.*, *M. retortiformis*) have neither (Berrill, 1931). The eggs of *M. pacifica* have very few test cells lying within a perivitelline space much smaller than that seen in typical urodele species.

The functions of the follicle cell layer vary among eggs from different ascidian species. The follicle cells of *Corella inflata* are filled with ammonia and cause the eggs to float (Lambert and Lambert, 1978). Highly vacuolated follicle cells of other species probably reduce the egg's specific gravity to slow the sinking rate of the egg (Harvey, 1927; Berrill, 1931). Kessel and Kemp (1962) described a secretory product in the follicle cells of *Ciona intestinalis* and *Molgula manhattensis*. However, these secretory masses break down during oocyte maturation and are not present at ovulation; at spawning, the eggs contain a granular material of unknown function. *M. pacifica* is the only species reported to have secretory follicle cells after ovulation and the only one whose follicle cells function in attachment. The cells themselves are essentially destroyed when the adhesive material is secreted.

Lucas (1927) reported that the eggs of *Molgula robusta* are often held together by strings of adhesive mucus which facilitate dispersal by reducing sinking rates. These strings could be wafted from the bottom of the culture dishes by stirring, a feat that would be impossible with the securely attached eggs of *M. pacifica.*

Kupffer (1875; cited in Berrill, 1931) argued that anural development was the forerunner of urodele development, and Lacaze-Duthiers (1877) classified anural and urodele species in separate genera. However, virtually all later authors consider direct development to be the derived state (Berrill, 1931). Anural development is probably polyphyletic (Berrill, 1931). It has arisen independently in at least two different families (Styelidae and Molgulidae), and within the Molgulidae it has probably arisen in at least four different clades (Berrill, 1931; Huntsman, 1922). Berrill (1931) argues convincingly that in each case anural development arose as an adaptation to sedimentary environments, where neither swimming nor habitat selection had a strong selective advantage. It follows then that the few anural species occurring on hard bottom represent reinvasions of the original habitat by species that had evolved the soft-bottom developmental mode. In *Molgula pacifica,* adhesive eggs may be one character that facilitated such a reinvasion and that allowed the species to recruit and maintain populations on hard bottoms in surgy habitats just as well as species with typical tadpole larvae.

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Preparation and Properties of Cnidocytes from the Sea Anemone *Anthopleura elegantissima*

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Abstract. Cnidocytes were isolated from the tentacles and acrorhagi of *Anthopleura elegantissima* by enzymatic treatment with papain followed by centrifugation in a Percoll-containing medium to produce a concentrated fraction of these cells. The morphology of the isolated cells, as revealed by light and electron microscopy, showed that these cells had intact plasma membranes and was comparable to that of cells *in situ*. Comparison of the ability of different substances to induce discharge in isolated and *in situ* cnidocytes showed that the responsiveness of isolated cells was reduced, but not abolished, compared to *in situ* cnidocytes. Electrophysiological recordings made from cnidocytes isolated from acrorhagi showed that these cells possess voltage-activated ionic currents, further proof that the isolation procedure did not effect the integrity of the plasma membrane. Discharge did not occur with changes in membrane potential.

Introduction

Cnidocyte is a collective term for the various types of "sting cells" used for prey capture, defense, and other functions by members of the phylum Cnidaria. Their common feature is a specialized product of the golgi apparatus, the cnida (or capsule or cnidocyst), which contains a coiled, inverted tubule. With appropriate stimulation the enclosed cnida discharges; the inverted tube rapidly everts into the external environment and either entangles the prey or penetrates it and injects a venom. The classification of cnidocytes is largely based upon the architecture of the cnida and its tubule and divides these cells into three major catagories: nematocytes, spirocytes, and ptychocytes (for reviews see Mariscal, 1974, 1984).

An understanding of the physiology of cnidocytes (for reviews see Picken and Skaer, 1966; Mariscal 1974), has been handicapped by the fact that *in situ* these cells frequently form part of a complex tissue. Thus it is difficult to separate the actions of the cnidocyte from those of the various accessory and supporting cells. The development of techniques for recovering isolated, intact cnidae from various sources (for review see McKay and Anderson, 1988), and the ease with which this can be done, made isolated cnidae the subject of numerous physiological and biochemical studies. These studies provide a detailed understanding of cnida structure, biochemistry, toxicology, and the mechanics of discharge. Isolated cnidae provide little useful information, however, on the physiological control of discharge since cnida discharge ultimately involves a stimulus to the cell's membrane which is absent in isolated cnida. Thus, a functional, intact membrane is necessary if a thorough understanding of the control of cnida discharge is to be achieved. Therefore, we were interested in developing methods with which to study cnida discharge using isolated, functional cnidocytes.

Previously we reported a method for isolating cnidocytes from *Cladonema* sp. (Hydrozoa). *Chrysaora quinquecirrha* (Scyphozoa), (Anderson and McKay, 1987), and *Hydra littoralis* (Hydrozoa) (McKay and Anderson, 1986, 1987) and described some of the electrophysiological properties of those cells. Here we report a substantial modification of that method that makes it possible to isolate large numbers of intact, functional cnidocytes from sea anemone tissues. We also compare the ability of isolated and *in situ* cnidocytes to discharge under a variety of conditions.

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Materials and Methods

Animals

Specimens of *Anthopleura elegantissima* were obtained from the Marine Science Institute, University of California, Santa Barbara, and were kept in flowing seawater at 15–18°C. Animals were fed twice weekly on a diet of *Artemia* nauplii, fish eggs, and shrimp.

Chemicals and solutions

Papain was obtained from Cooper Biochemicals and Percoll was purchased from Pharmacia. CHAPS (3-[3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate), deoxycholic acid, and taurocholic acid were purchased from Sigma. All other chemicals were reagent grade and obtained from Fisher Scientific. The different salines and their compositions were as follows: Ca-free artificial seawater (m*M*), NaCl, 476; KCl, 9.7; $MgCl_2$, 24; $MgSO_4$, 27; $NaHCO_3$, 3; HEPES, 10; pH 7.4, artificial seawater (m*M*) NaCl, 466; KCl, 9.7; $CaCl_2$, 10; $MgCl_2$, 24; $MgSO_4$, 27; HEPES, 10; pH 7.4 and concentrated artificial seawater (m*M*), NaCl, 1120; KCl, 22; $CaCl_2$, 23; $MgCl_2$, 65; $NaHCO_3$, 2.

Cnidocyte preparation

Animals were anesthetized in a 1:1 mixture of seawater (SW) and 0.37 *M* $MgCl_2$. When insensitive to touch, the tentacle ring was removed with scissors and transferred to a smaller vessel containing the same medium. Individual tentacles and acrorhagi were separated from other tissue and placed in 1.5 ml polypropylene microcentrifuge tubes. It is crucial that mucus produced during the dissection be removed and kept to a minimum.

Cnidocytes were freed from the surrounding tissues by enzymatic digestion with papain prepared as follows. 0.05 ml of a papain suspension in 50 m*M* sodium acetate buffer (28.8 $\mu g/\mu l$; 20.9 BAEE units/mg) was added to 0.95 ml SW followed by the addition of 0.2 mg of dithiothretol (DTT). The pH was adjusted to pH 7.5–7.6 with NaOH. The tissue was added to the enzyme solution and the volume adjusted to 1.5 ml with SW. The ratio of volumes of medium to volumes of loosely packed tissue was approximately 4:1. The tissue was then placed on an oscillating shaker whose speed was just sufficient to keep the tissue in motion. The progress of the digestion was followed by microscopic examination. Usually 1–1.5 hours were required to liberate large numbers of cnidocytes, although longer times were sometimes necessary. When the tissue was judged sufficiently dispersed, the whole digest was centrifuged on a Fisher Microcentrifuge equipped with swinging buckets for 3–4 minutes at an RCF of 1100 × *g*. This pelleted all of the dispersed cells without undue compaction. The supernatant was discarded and replaced with 0.72 ml of Percoll and 0.48 ml of concentrated artificial SW. The pellet was resuspended by gentle tapping and allowed to sit several minutes until most of the tissue had risen towards the surface. The tube was then centrifuged again at an RCF of 2000 × *g* for 5 minutes. Following centrifugation unwanted cells and debris remained in the upper portion of the medium. The supernatant was discarded and the pellet, composed almost exclusively of cnidocytes, was resuspended in a small volume of SW. In some cases it was necessary to resuspend the pellet and recentrifuge it in the Percoll-containing medium to remove excess debris.

Scanning electron microscopy (SEM)

Isolated cnidocytes were allowed to adhere to protamine sulfate-coated coverslips, rinsed to remove nonadhering cells and fixed as previously described (Anderson and Schwab, 1984). After fixation, specimens were critical-point dried with CO_2, sputter-coated with gold-palladium, and examined using a JEOL 35C.

Transmission electron microscopy (TEM)

Isolated cnidocytes were fixed using the same protocol as for SEM except that the isolated cnidocytes were fixed as a suspension. Solutions were exchanged by briefly centrifuging the cells into a pellet, replacing the supernatant, and then resuspending the pellet. After fixation the isolated cnidocytes were dehydrated through an ethanol series and propylene oxide and embedded in Epon. Prior to polymerization, the cells were centrifuged into a pellet to make it easy to locate them for thin sectioning. Sections were post-stained with uranyl acetate and lead citrate and examined with a JEOL 100 TEM.

Cnidocyte behavioral assay

The ability of isolated cnidocytes and *in situ* cnidocytes in the tentacles of *A. elegantissima* to discharge under a variety of conditions was assayed in the following manner. Excised tentacles were pinned to a layer of Sylgard (Dow Corning) in the bottom of a petri dish containing approximately 5 ml of SW or artificial saline. Isolated cnidocytes were allowed to settle on and adhere to coverslips coated with protamine sulfate. Test substances, in solution, were applied from a micropipette using brief (5–100 ms) pulses of N_2 (Picospritzer II, General Valve Co.). The pipette was positioned to within 10 μm of the cell being tested. With this system we ejected volumes from 20–840 × 10^{-9} l. The results were observed through a Hoffman Modulation Contrast microscope (total magnification = 320×).

Electrophysiological recordings

Electrophysiological recordings were obtained from cnidocytes isolated from the acrorhagi. Whole-cell patch clamp recordings were made using a Dagan 8900 Patch Clamp Amplifier equipped with a 0.1 Gohm headstage. Patch pipettes were filled with a high K^+, low Ca^{++} recording solution (Anderson, 1985). Leakage and capacative currents were removed from the records presented here by subtracting currents generated by equal and opposite voltage steps (for details of the methods, see Anderson and McKay, 1987).

Results

Nomarski micrographs of aliquots of cnidocytes are shown in Figure 1. The bulk of material is intact, isolated cnidocytes; the amount of debris is small. The majority of cnidocytes isolated from the tentacles (Fig. 1A) are spirocytes, although nematocytes are also present. The cnidocyte membranes appear intact and the cnida is visible within each cell. We were unable to separate the different types of cnidocytes from the tentacles as they all have a very low buoyant density beyond the density (d > 1.12 g/ml) we could conveniently achieve using Percoll solutions. Cnidocytes isolated from the acrorhagi are shown in Figure 1B. Again the cnida is visible within each cell. Most of the cell cytoplasm appears as a small girdle or bleb encircling the cnida.

The appearance of the isolated cnidocytes was better revealed using SEM. (Fig. 2). What is clearly a spirocyte is shown in Figure 2A. The membrane has collapsed around the tough cnida with its uneverted tube. The nematocyte (type unknown) in Figure 2B shows a protoplasmic extrusion (arrow) from its apical end; this may be a ciliary structure. Such structures were observed on only a few cells.

To further verify that the isolated cnidocytes were intact cells we examined their ultrastructure by transmission electron microscopy (Fig. 3). It should be stressed that these sections were obtained from pellets of isolated cnidocytes; hence, other cells are present. Figure 3 shows a nematocyte in cross-section. The cell's interior is dominated by the cnida. The inverted tube is embedded in an electron-dense material and is surrounded by the thick two-layered cnida wall, which in turn is surrounded by the cnida membrane. The nucleus and other cytoplasmic structures are visible also and all are enclosed by an apparently intact plasma membrane.

A spirocyte in longitudinal section is shown in Figure 3B. The cnida wall is single-layered and the internal surface has the serrated appearance characteristic of spirocytes (Picken and Skaer, 1966.). The cnida and other cytoplasmic constituents are once again surrounded by a plasma membrane.

Electrophysiology

Although electron microscopy showed that the isolated cnidocytes were intact, we made electrophysiological recordings to demonstrate that their membranes were functional. Because of their relatively large size we used cnidocytes isolated from the acrorhagi. Under voltage clamp (Fig. 4A), the total membrane currents evoked by step-depolarizing the cell from a holding potential of −70 mV through a range of depolarized membrane potentials consist of both inward and outward currents: a fast inward current followed by a more prolonged outward current. A current/voltage plot for the peak inward current (Fig. 4B) shows that the inward current activates at approximately 0 mV, reaches a maximum at +20 mV, and reverses at +65 mV. We did not study the ionic dependence of this or any other currents in these cells.

Observations on in situ *and isolated cnidocytes*

The treatments given to excised tentacles and isolated cnidocytes and their efficacy in evoking discharge are summarized in Table I. To assess the effect the extracellular medium may have had on a cnidocyte's ability to discharge, treatments were repeated in three different media: Ca-free ASW, normal SW, and 1:1 0.37 *M* $MgCl_2$ and SW. Because spirocytes comprise the majority of cnidocytes in anemone tentacles and can be unambiguously identified in the undischarged state, they were the only type of isolated cnidocyte tested. We did not attempt to identify the types of cnidocytes that discharged in the excised tentacle.

In situ cnidocytes bathed in normal SW invariably were discharged by 0.5 *M* KCl. Isolated cnidocytes bathed in normal SW responded only occasionally to 0.5 *M* KCl; there were no responses in the other two bathing media. When calcium was added to the 0.5 *M* KCl (final [Ca] = 0.17 *M*) *in situ* cnidocytes discharged in the Ca-free artificial seawater (ASW) as did a few of the isolated cnidocytes bathed in Ca-free ASW and normal SW.

Acid and alkaline SW were only minimally effective at eliciting discharge. Only *in situ* cnidocytes bathed in SW and "spritzed" with acidified SW (HCl, pH 2.5) discharged. To test for osmotic effects, distilled water and 1 *M* sucrose in SW were tested. Neither substance caused discharge. Since calcium chelating agents have been implicated in the discharge of isolated cnidae (Lubbock and Amos, 1981), we examined the effect of the sodium citrate and potassium citrate. Only potassium citrate evoked discharge and only in *in situ* cnidocytes bathed in normal SW.

To further examine the role of external ions in controlling discharge, we examined the efficacy of 0.5 *M* KCl in discharging *in situ* cnidocytes in bathing solutions of differing ionic compositions. In Na-free (N-methyl-D-

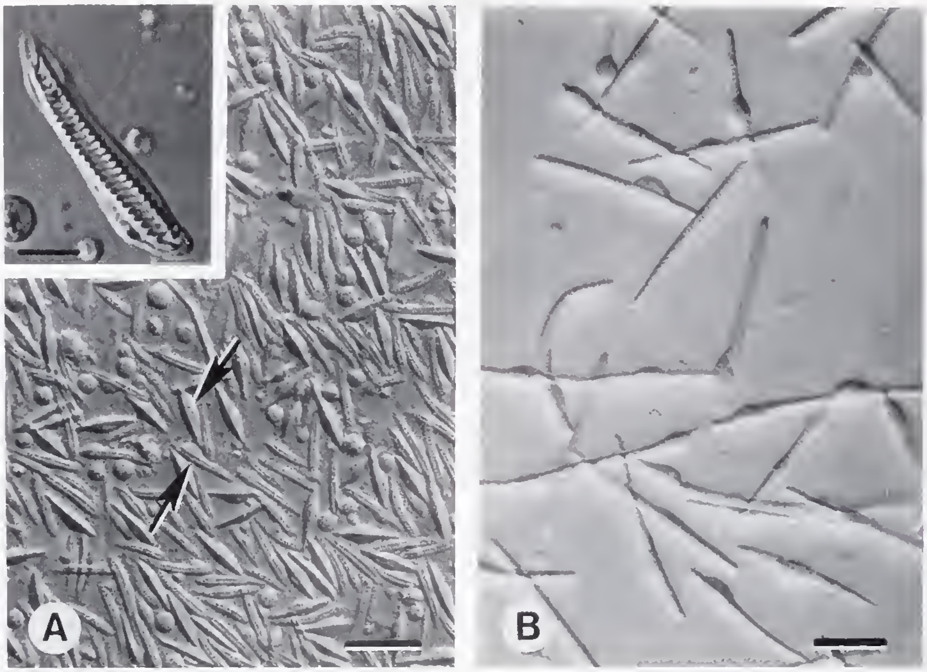

Figure 1. Nomarski micrographs of representative aliquots of cnidocytes isolated from tentacles and acrorhagi of *Anthopleura elegantissima.* (A) Isolation of cnidocytes from tentacles produced a mixture of cnidocyte types. Arrows show different kinds of cnidocytes (scale = 25 μm). Insert is a higher magnification view of a spirocyte isolated from the tentacles. The coiled tubule of the cnida is clearly visible (scale = 5 μm). (B) Cnidocytes isolated from acrorhagi (scale = 50 μm).

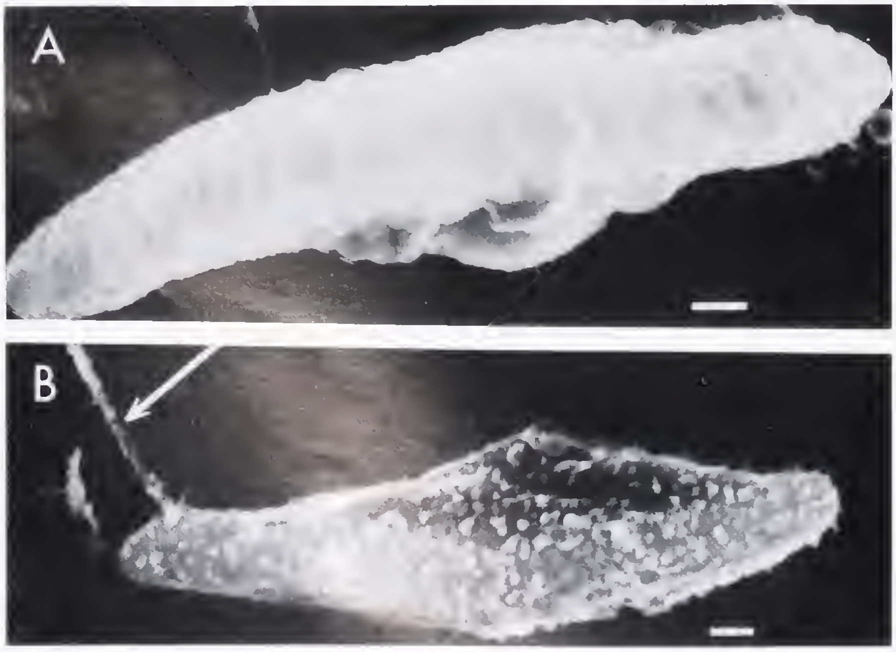

Figure 2. Scanning electron micrographs of two different types of cnidocytes isolated from the tentacles. (A) Spirocyte. (B) Nematocyte, type unknown. The cytoplasmic projection (arrow) may be a ciliary structure (A, B, scale = 1 μm).

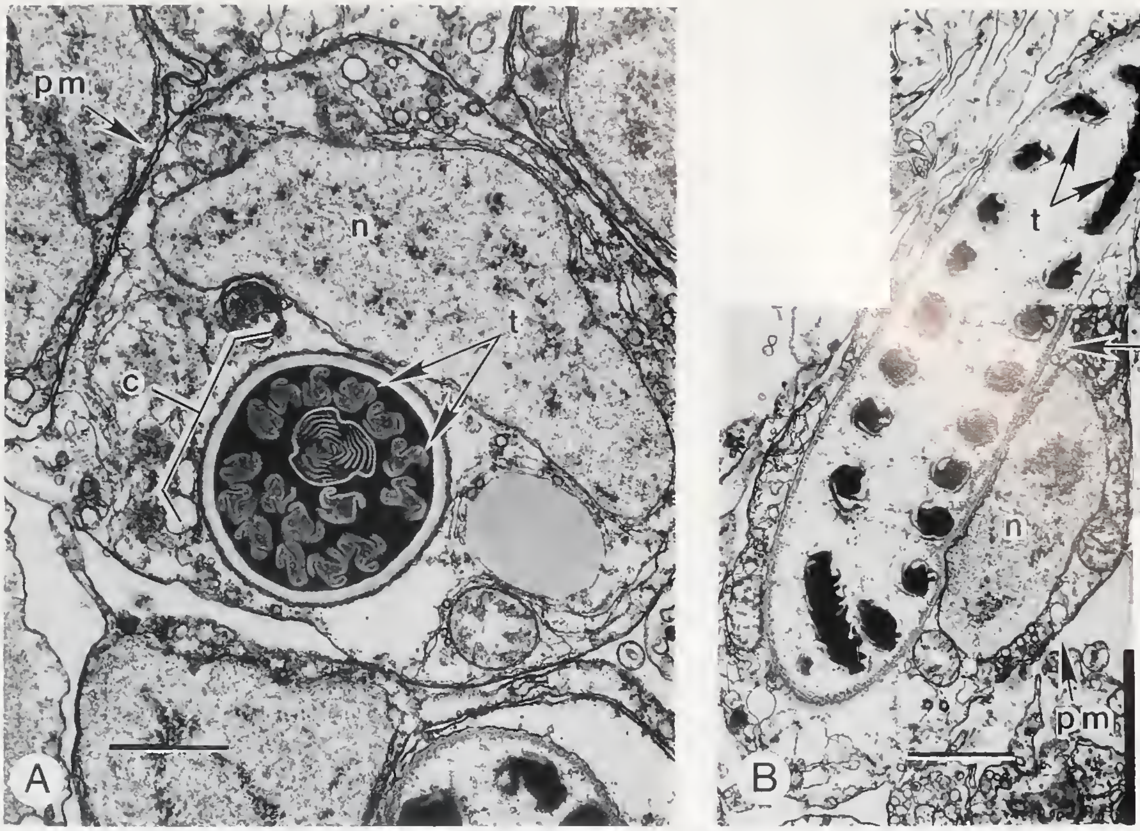

Figure 3. Transmission electron micrographs of different types of cnidocytes isolated from the tentacles of *Anthopleura elegantissima.* The fixed cells were embedded as pellets. (A) A cross-section of a nematocyte containing an undischarged cnida in its cytoplasm (scale = 1 μm). (B) Longitudinal section through a spirocyte with an undischarged cnida and other cytoplasmic organelles (scale = 2 μm). c, cnida; cw, capsule wall; n, nucleus; pm, plasma membrane; t, tubule.

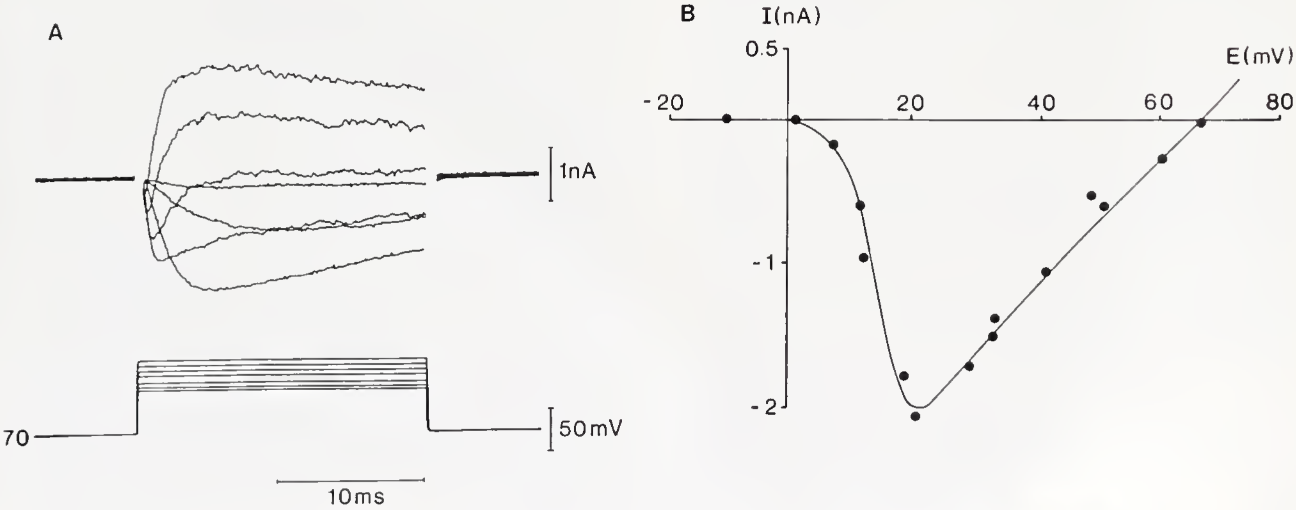

Figure 4. Voltage-clamp recordings from a cnidocyte isolated from the acrorhagi of *Anthopleura elegantissima.* (A) Record of the total membrane currents produced when the cell was step depolarized from a holding potential of −70 mV to −20 mV and then in 7.5 mV increments. (B) Current-voltage plot for the peak inward currents obtained from (A).

Table I

Comparison of the ability of different substances to discharge isolated and in situ *cidocytes from* Anthopleura elegantissima. *Whole tentacles (WT) and isolated spirocytes (IS) were tested in three different bathing solutions*

	WT	IS	WT	IS	WT	IS
Bathing medium	Normal SW		Ca-free ASW		(1:1) 0.34 M $MgCl_2$/SW	
Test solution						
0.5 M KCl	+	+/−	−	−	−	−
0.5 M KCl + 0.17 M $CaCl_2$	+	+/−	+	+/−	−	−
SW pH 2.5	+	−	−	−	−	−
SW pH 10.5	−	−	−	−	−	−
Potassium citrate 100 mM	+	−	+	−	nt	nt
Sodium citrate 100 mM	−	nt	−	nt	nt	nt
Taurocholic acid 25 mM	+	+	+	+	+	+
Deoxycholic acid 25 mM	+	+	+	+	+	+
CHAPS 25 mM	+	+	+	+	+	+
1 M sucrose in SW	−	−	−	−	−	−
Distilled water	−	−	−	−	−	−
Brine shrimp homogenate	−	−	−	−	−	−
Fish skin homogenate	−	−	nt	nt	nt	nt

Key: + discharge; − no discharge; nt not tested.

glucamine substituted) or Mg-free (Na substituted) ASW, discharge occurred in response to applied KCl. Discharge to applied KCl was, however, inhibited by the addition of the potassium channel blocker, 4-aminopyridine (7.5 mM) to a bathing solution of normal SW, implying that potassium is acting through blockable channels.

The only substances that consistently elicited discharge in both isolated and *in situ* cnidocytes in all media were derivates of the cholic acids (CHAPS, taurocholic and deoxycholic acids). These substances have been used classically to demonstrate the competence of cnidocytes (Pantin, 1942) and isolated cnidae (Burnett *et al.*, 1968) to discharge, but considering the strong lipid solubilizing nature of these substances it is not surprising that they are effective discharge inducing agents.

To investigate more physiologically realistic stimulants, homogenates of *Artemia* nauplii and fish-skin (*Mugil cephalis*) were prepared by sonicating these tissues in SW. The filtrates of these substances had no effect on either isolated or *in situ* cnidocytes. (Table I).

Discussion

We have developed a method for isolating intact, functional cnidocytes from the tentacles and acrorhagi of a sea anemone using enzymatic dissociation. This procedure enabled us to isolate intact cnidocytes in a fraction that was essentially free of other types of cells. The technique appears to be generally applicable to cnidocyte-bearing tissues. Using the same basic method, with modifications, we have been able to dissociate cnidocytes from a variety of other cnidarians, including *Aiptasia* sp., *Bunodosoma cavernosa, Calliactis tricolor* (Anthozoa) (unpub. results), *Chrysaora quinquecirra* (Scyphozoa), *Cladonema* sp., and *Hydra littoralis* (Hydrozoa) (McKay and Anderson, 1986, 1987; Anderson and McKay, 1987).

Enzymatic dissociation of cnidocyte-bearing tissues has been attempted previously. Pepsin (Glaser and Sparrow, 1909), papain (Phillips and Abbott, 1957), and trypsin (Yanagita, 1959) were all reported to be either ineffective or to have a deleterious effect on the condition of the cnidae. The reason for the difference in success is unclear, but it could be attributable to the type of enzymes used since we found that papain from different sources produced variable results; some were essentially ineffective while others produced only isolated cnidae.

A variety of approaches were used to determine whether the isolated cells were intact and to distinguish between complete cells and isolated cnidae. Light microscopy clearly demonstrated that the bulk of the isolated tissue was composed of cnidocytes, as defined by the presence of the cnida. In many cases (Fig. 1A, insert) organelles, particularly nuclei, could be seen between the cell membrane and the cnida, suggesting that the cells were intact. The presence of the cell membrane and various organelles was confirmed in electron micrographs of thin sections of pellets of isolated cnidocytes (Fig. 3). The apparent absence of ciliary cones on our isolated cnidocytes was worrisome and may reflect damage during the isolation procedure. In anthozoans, however, ciliary cones occur only on nematocytes and not spirocytes (Robson, 1973; Mariscal *et al.*, 1976) so their absence on all cnidocytes is not unexpected.

The presence of the cell membrane and other organelles suggests that the cells are intact but ultrastructural techniques provide only limited information on the health of the cell. Although the isolated cnidocytes retained the ability to discharge, this is not proof that they are in good condition because isolated cnidae can discharge. The fact that the isolated cells supported transmembrane ionic currents (Fig. 4A) and negative resting potentials is good evidence that their membranes were intact and functional and not damaged by the isolation procedure.

A further reason for conducting electrophysiological experiments with these cells was to determine whether discharge could be elicited by electrical activity. A common means of effecting discharge from isolated cnidae and intact tissues is with electrical stimulation (Lubbock *et al.*, 1981). Previous work (Anderson and McKay, 1987) on cnidocytes from hydrozoans and scyphozoans has shown, however, that cnida discharge cannot be effected by changes in membrane potential nor by the presence or selective blockade of any of the ionic currents present in the cell. The fact that none of the cells examined electrophysiologically in this study discharged during the recordings indicates that this finding can be extended to the anthozoans. The reason why electrical stimulation is such an effective means of evoking cnida discharge is, therefore, unclear although it is possible that the type of electrical stimuli required to elicit discharge may be physiologically unrealistic.

The difficulties of inducing spirocyte discharge has been noted before (Robson, 1973) and our results agree with this, but taken together, our results show that although the responsiveness of isolated cnidocytes to stimuli is greatly diminished compared to *in situ* cnidocytes, it is not abolished. This parallels the sensitivity of isolated cnidae as compared to *in situ* cnidocytes (Blanquet, 1970).

Previous work (Conklin and Mariscal, 1976) on the responsiveness of cnidocytes *in situ* has shown that cnidocyte discharge requires a combination of chemical and mechanical stimuli. In that type of study, the typical approach was to coat a glass rod or coverslip with the chemical stimulant and then to place it in contact with the cnida-bearing tissue, thus providing both a chemical and mechanical component to the stimulus. However, working with single cells meant that we could not use the traditional methods of mechanical stimulation. Instead we attempted to stimulate the cells by spritzing a known chemical at the cell; the force of ejection served as the mechanical component. The use of brief, discrete episodes of stimulation reduced the risk of receptor desensitization from prolonged exposure to the stimulant. Although we could quantify the chemical component of the stimulus, this could not be done with the mechanical component. Indeed, a poor mechanical stimulus may explain the failure of the brine shrimp or fish skin homogenates to discharge either the *in situ* or isolated cells since both these tissues, when intact, are readily captured by the tentacles of intact anemones.

While the low incidence of discharge could be partly attributable to the quality of the mechanical components of the stimulus, the fact that so few isolated cnidocytes discharged may also mean that *in situ* their discharge is regulated by adjacent cells and nerves, as has been proposed elsewhere (Westfall *et al.* 1971; Westfall, 1973; Hufnagel *et al.*, 1985).

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A Physiological Comparison of Bivalve Mollusc Cerebro-visceral Connectives With and Without Neurohemoglobin. I. Ultrastructural and Electrophysiological Characteristics

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Abstract. The presence of hemoglobin in tissues of a small number of species is puzzling when homologous tissues of closely related species do not possess hemoglobin. Several species of bivalve molluscs possess nervous systems with nerve hemoglobin (neurohemoglobin). These systems were compared to nervous systems from other bivalve molluscs without neurohemoglobin to determine ultrastructural and electrophysiological characteristics under normoxic conditions in an attempt to locate any differences between these two types of nervous systems. Cerebro-visceral connectives from the bivalves *Tellina alternata* and *Spisula solidissima* with neurohemoglobin and *Tagelus plebeius* and *Geukensia demissa* without neurohemoglobin possess a perineural sheath, a subjacent peripheral layer of glial cells, and glial cell processes that enwrap bundles of 0.2–0.4 μm diameter axons. Neurohemoglobin-containing cerebro-visceral connectives have smaller axon bundles and more dense perineural sheaths than those without neurohemoglobin. These features may be important in oxygen delivery from the neurohemoglobin to the axons. Action potential traces, conduction velocities, refractory periods, strength-duration relationships, and temperature responses of all four connectives are typical of nerves possessing very small axons. There are no obvious electrophysiological differences between cerebro-visceral connectives with and without neurohemoglobin.

Introduction

Tissue hemoglobins are distributed sporadically throughout most of the biological taxa. In general, hemoglobins may participate in the facilitated diffusion of oxygen or they may serve to store oxygen for use during periods of low oxygen availability (Colacino and Kraus, 1984; Burr and Harosi, 1984; Kraus and Colacino, 1986). Because hemoglobin can occur in a particular tissue of one or a few species of animals and not in homologous tissues of the majority of closely related species, the question arises as to why the need for hemoglobin exists. If hemoglobin is needed to augment the oxygen supply to a specific tissue, why are the oxygen demands of the homologous tissues with and without hemoglobin different? What, if anything, is different about the structure, physiology, or biochemistry of each tissue?

The hemoglobin-containing nerves of marine invertebrates represent an ideal system for this type of study for several reasons. First, although the nervous systems of several organisms tolerant of anoxic conditions can respond to stimulation in the absence of oxygen (Mangum, 1980; Surlykke, 1983), nervous tissue in general is functionally dependent on aerobic metabolism and thus on oxygen availability. In addition, nervous function can be readily monitored. Second, nerve hemoglobins have been noted in many invertebrates from several phyla, but it is by no means common (Wittenberg *et al.,* 1965). Therefore, it is possible to locate species without nerve hemoglobins which are related to species with nerve hemoglobins for comparative study.

The connectives between ganglia in the bivalve molluscs are relatively simple in structure and function. They are distinct anatomical structures, self-contained within a protective sheath, and are thus easily prepared for ultrastructural and electrophysiological study. Moreover, tissue function can be monitored unambiguously

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because the axons are relatively homogeneous in type and size. The cerebro-visceral connectives, which are the main routes for communication between the cerebropleural and visceroparietal ganglia, are relatively long in bivalves with an elongated anteroposterior axis and thus were selected for this work.

Cerebro-visceral connectives from several bivalve molluscs lacking hemoglobin have been examined with respect to ionic and osmotic stresses (Willmer, 1978a, b), ionic permeability (Satelle and Howes, 1975), and ultrastructural organization (Gupta *et al.*, 1969). Kraus and Colacino (1986) reported that neurohemoglobin in bivalve cerebro-visceral connectives may serve as an oxygen supply for nervous activity during anoxic conditions. In this paper, basic ultrastructural and electrophysiological characteristics of the cerebro-visceral connectives from the bivalves *Tellina alternata* and *Spisula solidissima,* which are bright red with neurohemoglobin, and the white cerebro-visceral connectives of the bivalves *Tagelus plebeius* and *Geukensia demissa,* which lack neurohemoglobin, were determined under normoxic conditions in an attempt to locate any differences that may exist between these two types of connectives. The first three bivalves belong to the same order while *G. demissa* belongs to a separate subclass.

Materials and Methods

Specimens of *Tellina alternata* and *Tagelus plebeius* were collected from the ebb tide level of a backwater sandy mud flat at Long Beach, North Carolina. Specimens of *Geukensia demissa* were collected at the flood tide level from a *Spartina* bed bordering the same mud flat. Specimens of *Spisula solidissima* were collected offshore and supplied by the Rutgers Shellfish Research Laboratory, Port Norris, New Jersey, and the United States Department of Commerce-NOAA, Milford, Connecticut. Most animals were obtained during the spring and summer.

The specimens were maintained in aerated seawater aquaria, 35‰ salinity, at 15–20°C. They were fed weekly with fish food (TetraMin, Carolina Biological) that was homogenized and stirred into the tank. Animals were observed siphoning the suspended food particles and food was found inside the animals' digestive diverticula during dissection. Their activity level and overall condition appeared stable for at least two months. Animals were usually used for experimentation within four weeks of collection. Animal sizes and weights are listed in Table I.

Dissection of cerebro-visceral connectives

Clams were opened by severing the adductor muscles, and held open for dissection with rubber bands in a glass bowl containing cold seawater. The siphons, gills, and palps were removed to expose the visceral and cerebral ganglia. An entire single cerebro-visceral connective was exposed by gently teasing away the embedding body wall musculature, taking great care not to stretch the connective or puncture the sheath. Once exposed, the connective was severed at the base of the ganglia on both ends and removed for experimentation. It was unnecessary to ligature the severed ends because the dense cellular matrix and tough perineural sheath prevented the neural contents from exuding out of the cut ends. One or both of the connectives were used from each animal. Entire connectives ranged from 2–4 cm to 6–8 cm in length and from 0.01 cm to 0.025 cm in diameter (*T. alternata* and *S. solidissima,* respectively). With some practice, the operation could be completed within 30 min.

Light and transmission electron microscopy

The cerebro-visceral connective, at approximately ¼ its length posterior to the cerebral ganglion, is free from branches and lies loosely inside the body wall. Connective segments, 0.5 to 1 cm in length, were excised from this location and fixed immediately in 2.5% glutaraldehyde in 0.2 *M* Millonig's phosphate buffer (pH 7.6) and 0.34 *M* NaCl for 0.5 h. After rinsing twice in Millonig's buffer, the segments were postfixed for 0.5–1.0 h in 2% OsO_4 in Millonig's phosphate buffer (pH 7.6). Following postfixation, specimens were dehydrated through a graded series of ethanols and embedded in Polybed 812 using propylene oxide as the infiltration solvent.

Thin sections were cut with diamond knives on a LKB Ultratome Nova at various positions along the length of the fixed connective segments. Sections were collected on bare copper hexagonal mesh grids, stained with alcoholic uranyl acetate and aqueous lead citrate, and photographed with a Phillips EM 300 transmission electron microscope. Electron micrographs of thin sections (5–10 sections from each of 4–5 individuals of each species) were used to determine the following distances and counts: connective diameter, perineural sheath thickness, axon density, axon size distribution, number of axons per bundle, and the greatest distance between the central axon and nearest glial cell within each bundle.

Electrophysiological measurements

To measure electrophysiological properties, an anterior segment of the cerebro-visceral connective, measuring approximately 2.5 cm in length, was excised and pipetted into a temperature-controlled nerve chamber (Fig. 1). While submerged in the chamber, the connective was positioned to lie across 12 platinum electrodes. The bathing fluid was slowly asperated beneath the electrodes until they suspended the moist connective in air. The chamber floor was covered with moistened lens pa-

Table I

Animal sizes and weights

Parameter	*Tellina alternata*	*Spisula solidissima*	*Tagelus plebeius*[a]	*Geukensia demissa*[a]
Maximum length, cm	5.0 ± 0.3[b]	7.8 ± 0.4	8.4 ± 0.3	10.1 ± 0.3
Fresh tissue weight, g[c]	3.4 ± 0.7	20.8 ± 3.6	14.7 ± 0.8	9.2 ± 1.7
Valve weight, g	5.3 ± 1.2	26.7 ± 3.4	9.8 ± 0.9	29.5 ± 8.9

[a] Species without neurohemoglobin.

[b] Values are given as mean ± standard deviation of 8–12 individuals.

[c] Tissue was removed from valves and blotted dry before weighing.

per to prevent dehydration of the connective and the chamber was sealed with a glass lid. Water did not condense on the connective during experimentation. All experiments were performed under normoxic conditions.

The connective was stimulated externally with a square wave stimulator (Grass SD9). The resultant compound action potential and stimulus pulse were monitored with a preamplifier (Narco) and microcomputer (Apple IIe) equipped with dual trace oscilloscope hardware and software (RC Electronics). Action potential traces were used to make the following measurements: action potential amplitude, conduction velocity, absolute and relative refractory periods, and the strength-duration relationship. Measurements were made at three temperatures after a 30 min equilibration period at each temperature (5, 10, and 15°C for *S. solidissima* and 10, 15, and 20°C for the others). Several electrophysiological parameters were determined with each preparation. Measurements were made with preparations from 5–8 individuals of each species.

Ultrastructural and electrophysiological data were analyzed using hierarchical analysis of variance to determine if variation could be attributed more to differences among individual species or between species with and without neurohemoglobin.

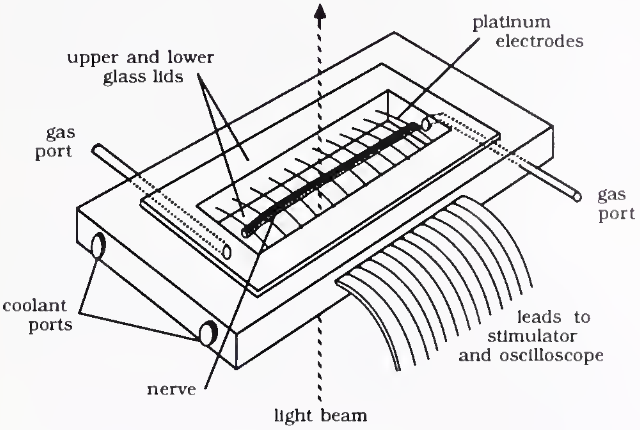

Figure 1. The nerve-chamber gas slide used for electrophysiological measurements.

Results

Neuroanatomy and ultrastructure

The bilaterally symmetrical nervous systems of lamellibranch bivalves typically have three pair of ganglia (visceroparietal, cerebropleural, and pedal), a number of major nerve trunks and connectives, and several finer nerve branches. The entire nervous system is contained within a tough, thin connective tissue-like sheath. The neuroanatomy of all four species of bivalves in this study is typical of lamellibranch bivalves with elongated anteroposterior axes (see Fig. 24.2, Bullock, 1965). However, the nervous systems differ in one regard: those of *Tellina alternata* and *Spisula solidissima* are vivid red while those of *Tagelus plebeius* and *Geukensia demissa* are unpigmented or white. The red color results from the presence of neurohemoglobin in the glial cells of the connectives (Kraus and Colacino, 1986; Doeller and Kraus, 1988).

The general anatomical design is similar among the cerebro-visceral connectives from all species examined. The connectives are surrounded by a 1–3 μm thick, fairly uniform acellular sheath that resembles the neural lamella of other invertebrate nervous systems (Horridge and Bullock, 1965). Situated subjacent to the sheath are glial cell bodies. Processes of glial cells ramify throughout the connectives and ensheath bundles of axons.

Ultrastructurally, the perineural sheaths of connectives with neurohemoglobin possess 20–30 tightly arranged concentric laminae consisting of a thick, electron-dense extracellular matrix material (Fig. 2). The individual laminae appear closer together, thinner, and more complete at the inner margin of the sheath. The outer laminae are about 60–70 nm apart. In contrast, the sheaths of connectives without neurohemoglobin consist of loosely arranged, thin laminae (Fig. 2) and resemble the cerebro-visceral connective sheaths of *Anodonta cygnea* (Gupta *et al.*, 1969) and *Mytilus edulis* (Willmer,

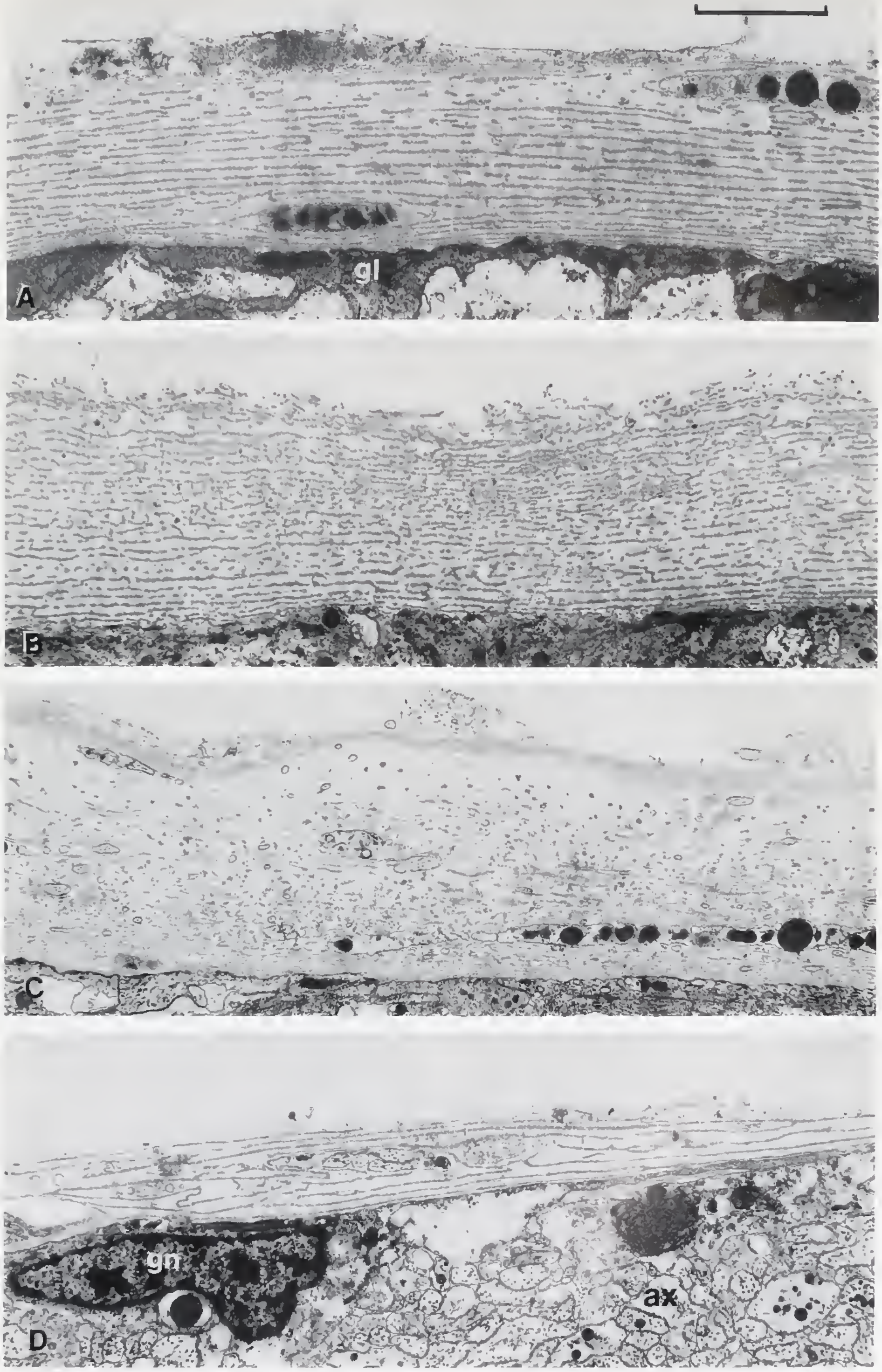

Figure 2. The neural sheaths of bivalve cerebro-visceral connectives. A. *Tellina alternata.* B. *Spisula solidissima.* C. *Tagelus plebeius.* D. *Geukensia demissa.* Bar is 2 μm for A–D. Glial cell, gl; glial cell nucleus, gn; axons, ax.

Table II

Morphometric characteristics of the cerebro-visceral connectives

Parameter	*Tellina alternata*	*Spisula solidissima*	*Tagelus plebeius*[a]	*Geukensia demissa*[a]
Connective diameter, μm	126 ± 21.0[b]	231 ± 9.7	169 ± 10.2	212 ± 35.4
Sheath thickness, μm	2.64 ± 0.74	3.58 ± 1.20	3.12 ± 0.50	2.01 ± 0.61
Axons/bundle[c]	72 ± 32	54 ± 40	177 ± 86	>250[d]
Cross sectional area of axon bundle, μm^2	5.16[e]	9.57	13.1	>36.6[d]
Maximum distance from axon to glial cell, μm	1.23 ± 0.50	1.44 ± 0.84	1.66 ± 0.45	≥6.7[d]
Density axons/μm^2 (axons counted)	6.18 ± 3.61 (7572)	2.12 ± 1.27 (2712)	4.50 ± 0.67 (5305)	3.67 ± 0.84 (4675)
Axons/connective (approximate)	73,000[f]	89,000	101,000	129,000
Volume of axons, % of connective	44.3[g]	37.6	36.2	53.8
Axon membrane, cm^2/g wet weight of connective	56,000[g]	30,000	43,000	47,000

[a] Species without neurohemoglobin.

[b] Unless specified, values are given as mean ± standard deviation of measurements obtained from 5–10 sections from 4–5 individuals.

[c] $P < 0.025$ between connectives with and without neurohemoglobin, using hierarchical ANOVA.

[d] Axon bundles were larger than electron micrographs.

[e] Calculated from axons/bundle and mean axon diameter (Fig. 6).

[f] Calculated from axon density and connective diameter.

[g] Calculated from axons/connective and mean axon diameter.

1978b), both which most likely lack neurohemoglobin. Fibers that run parallel to the long axis of the connectives are scattered between the laminae in all sheaths examined.

General morphometric characteristics are listed in Table II. Connectives with and without neurohemoglobin have similar axon density and relative axon volume. They possess a large axon membrane surface area, similar to the garfish olfactory nerve with 65,000 cm^2/g (Easton, 1971). In addition, there is no significant difference in sheath thickness between cerebro-visceral connectives with and without neurohemoglobin. However, axon fields in connectives with neurohemoglobin are more subdivided by glial cell processes than axon fields in connectives without neurohemoglobin, thus connectives with neurohemoglobin possess significantly fewer axons per bundle (Fig. 3; Table II). Cerebro-visceral connectives without neurohemoglobin from species in this study and from *Mytilus edulis* possess large, densely packed axon fields, relatively uninterrupted by glial cell processes (Fig. 3; Willmer, 1978b). This difference in architecture results in smaller axon bundles and shorter mean diffusion distances between axons and glial cells in neurohemoglobin-containing connectives (Fig. 3; Table II).

Glial cells in red connectives possess a granular, electron-dense cytoplasm (Fig. 3), presumably due to the presence of neurohemoglobin (Ruppert and Travis, 1983; Doeller and Kraus, 1988). Prior and Lipton (1977) also report a granular, electron-dense cytoplasm in the glial cells of *S. solidissima* siphonal nerves. In contrast, the cytoplasm of glial cells in unpigmented connectives appears much less dense (Fig. 3).

In all four cerebro-visceral connectives, more than 75% of the axons fall within one standard deviation of the mean axon diameter for each species (Figs. 4, 5). Although the larger connectives tend to have larger mean axon diameters (Fig. 5), the differences in mean axon diameter cannot be attributed to the presence of neurohemoglobin. The mean cerebro-visceral axon diameter (0.432 μm) of the mussel *G. demissa* (Fig. 5) is very similar to the mean cerebro-visceral axon diameter (0.403 μm) of the mussel *Mytilus edulis* (Willmer, 1978b).

Electrophysiology

Examples of externally recorded compound action potentials from cerebro-visceral connectives at different temperatures are shown in Figure 6. Action potentials conducted in either direction were indistinguishable in a given preparation, thus other electrophysiological characteristics were measured without regard to conduction direction. Action potential traces typically consisted of a stimulus artifact, a few fast, low-amplitude waves, and a

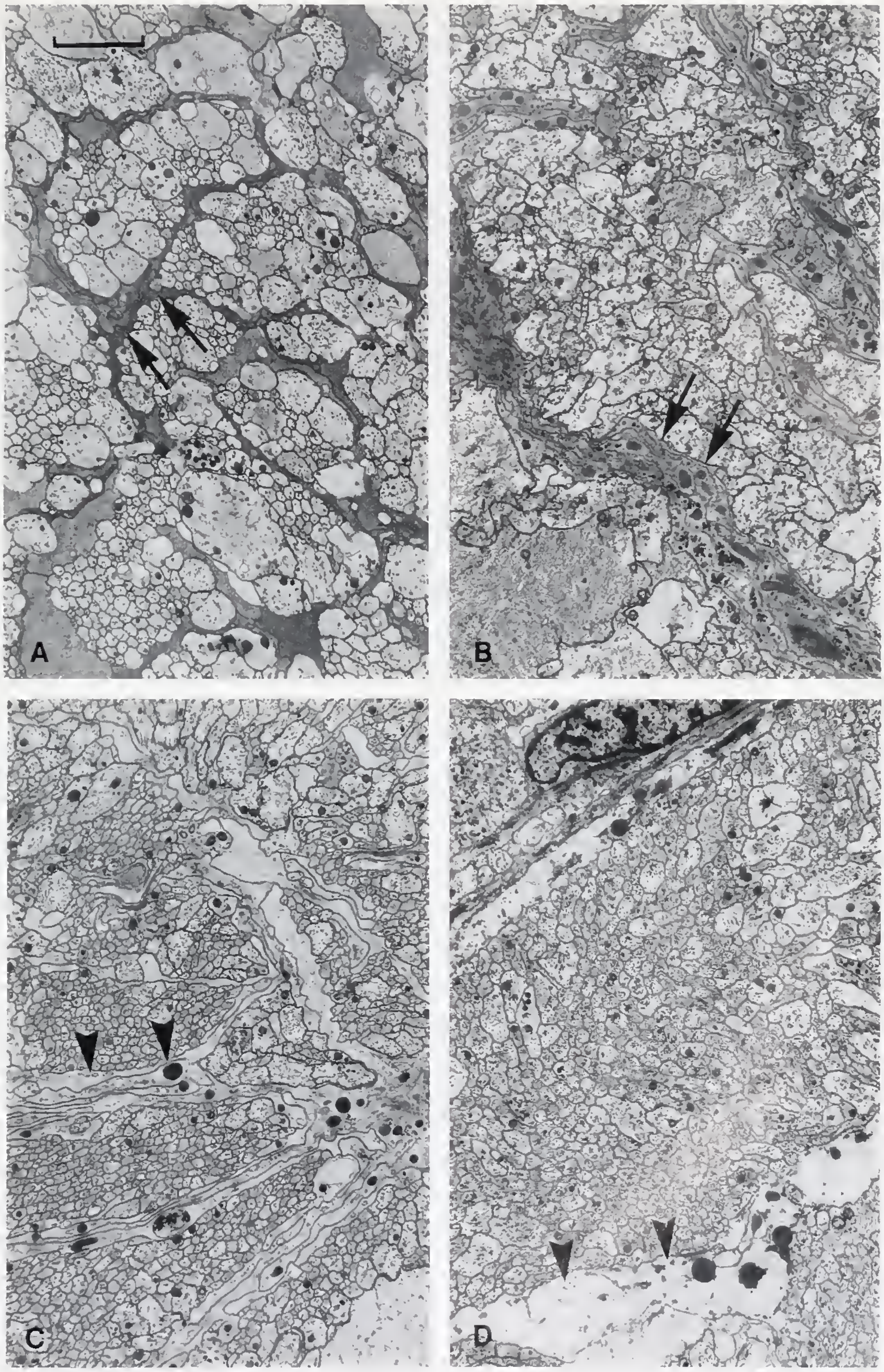

Figure 3. Axon bundles of bivalve cerebro-visceral connectives. A. *Tellina alternata.* B. *Spisula solidissima.* C. *Tagelus plebeius.* D. *Geukensia demissa.* Bar is 2 μm for A–D. Note the neurohemoglobin-containing glial cells (arrows) of *Tellina alternata* and *Spisula solidissima* and the neurohemoglobin-less glial cells (arrowheads) of *Tagelus plebeius* and *Geukensia demissa.*

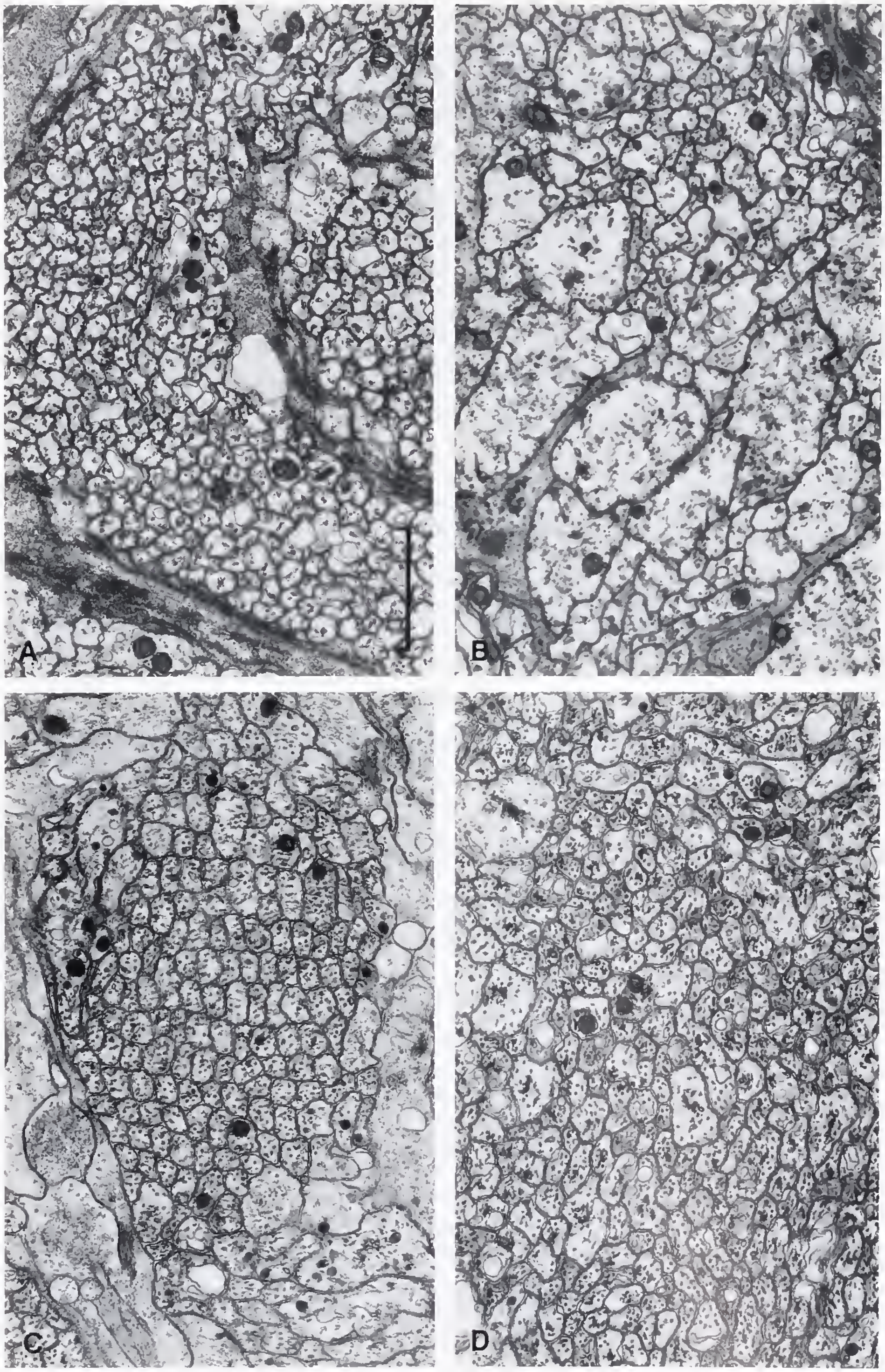

Figure 4. Axons of bivalve cerebro-visceral connectives. A. *Tellina alternata* B. *Spisula solidissima* Note the presence of several larger diameter axons. See Figure 6 for axon size histograms. C. *Tagelus plebeius*. D. *Geukensia demissa*. Bar is 1 μm for A–D. The tightly packed axons in all connectives leave little extracellular space.

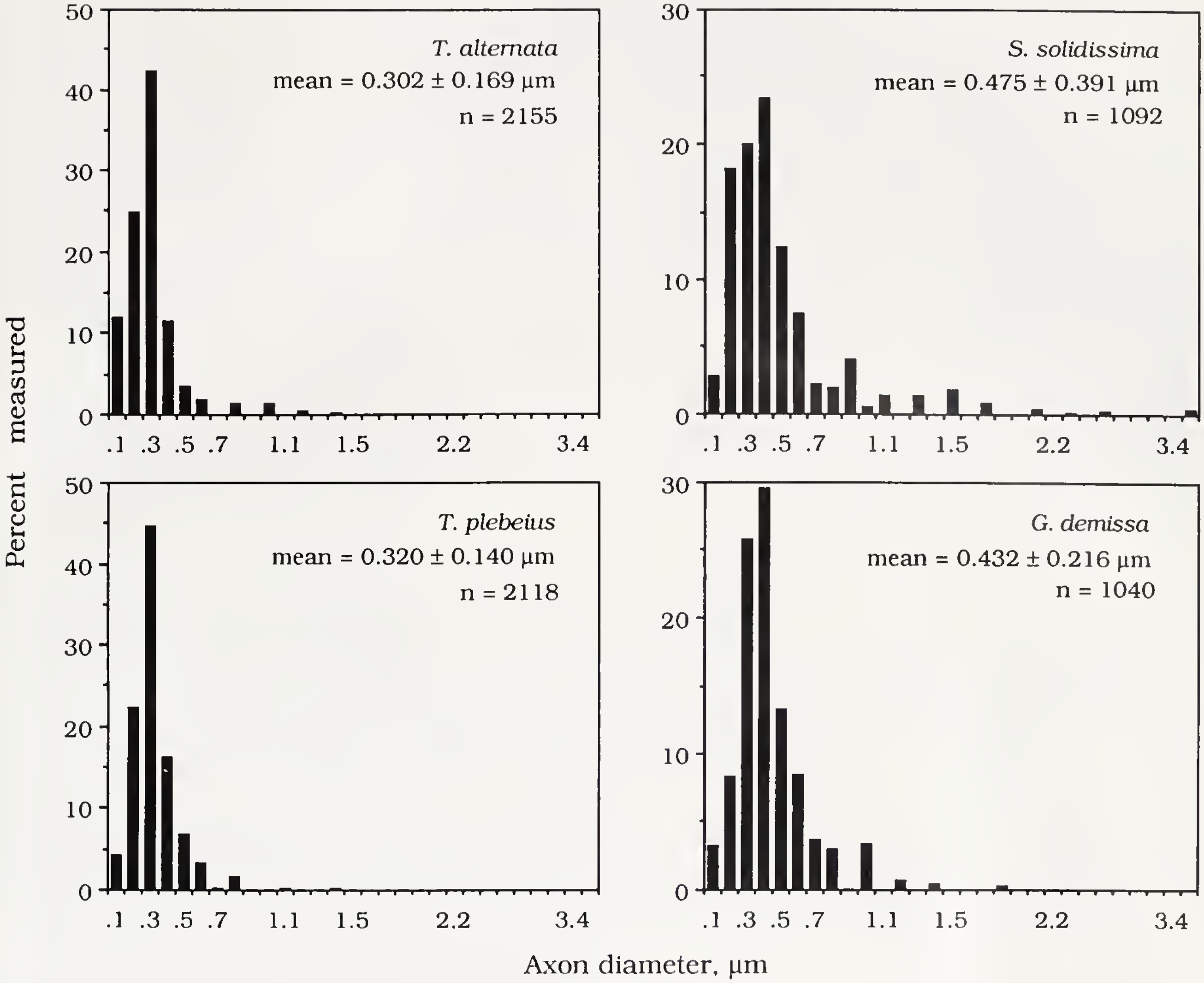

Figure 5. Axon size distributions of bivalve cerebro-visceral connectives. The mean axon diameter (± standard deviation) is given for each species, n is the number of axons measured. Axon diameters were sorted into 0.1 μm divisions for axons 0.1–0.8 μm diameter and 0.2 μm divisions for axons above 0.8 μm diameter. Axons oblong in cross-section were assigned an average diameter based on their long and short diameter.

slow, large-amplitude biphasic wave (Fig. 6). The slow, large-amplitude wave presumably represented action potentials conducted by the majority of small diameter (0.2–0.5 μm) axons. The faster low-amplitude waves preceding the major spike presumably represented action potentials from a small group of fibers greater than 0.5 μm in diameter. A small number but significant volume of the axons from *S. solidissima* connectives are 1.5–3.5 μm diameter (Figs. 4, 5) and this is reflected in the pronounced higher conduction velocity spike (Fig. 6). All electrophysiological measurements were made with the predominant slower velocity spike.

At near-habitat temperatures (10°C for *S. solidissima* and 20°C for the others), the maximum stimulus voltage of all connectives for total recruitment was 2–4 V at 1 ms duration. Externally recorded spike amplitudes at maximum stimuli measured 2–8 mV. Willmer (1978b) reported a 6–10 V spike amplitude in *Mytilus edulis* cerebro-visceral connectives, measured with sucrose gap. Spike duration lasted 15–30 ms at a conduction distance of about 10 mm. Generally, the peak amplitude decreased by about 50% per centimeter length of connective conducted, presumably as the separate components of the compound action potentials became temporally dispersed.

Conduction velocities were measured at peak action potential amplitude over conduction distances of 4–20 mm between the stimulating and recording electrodes

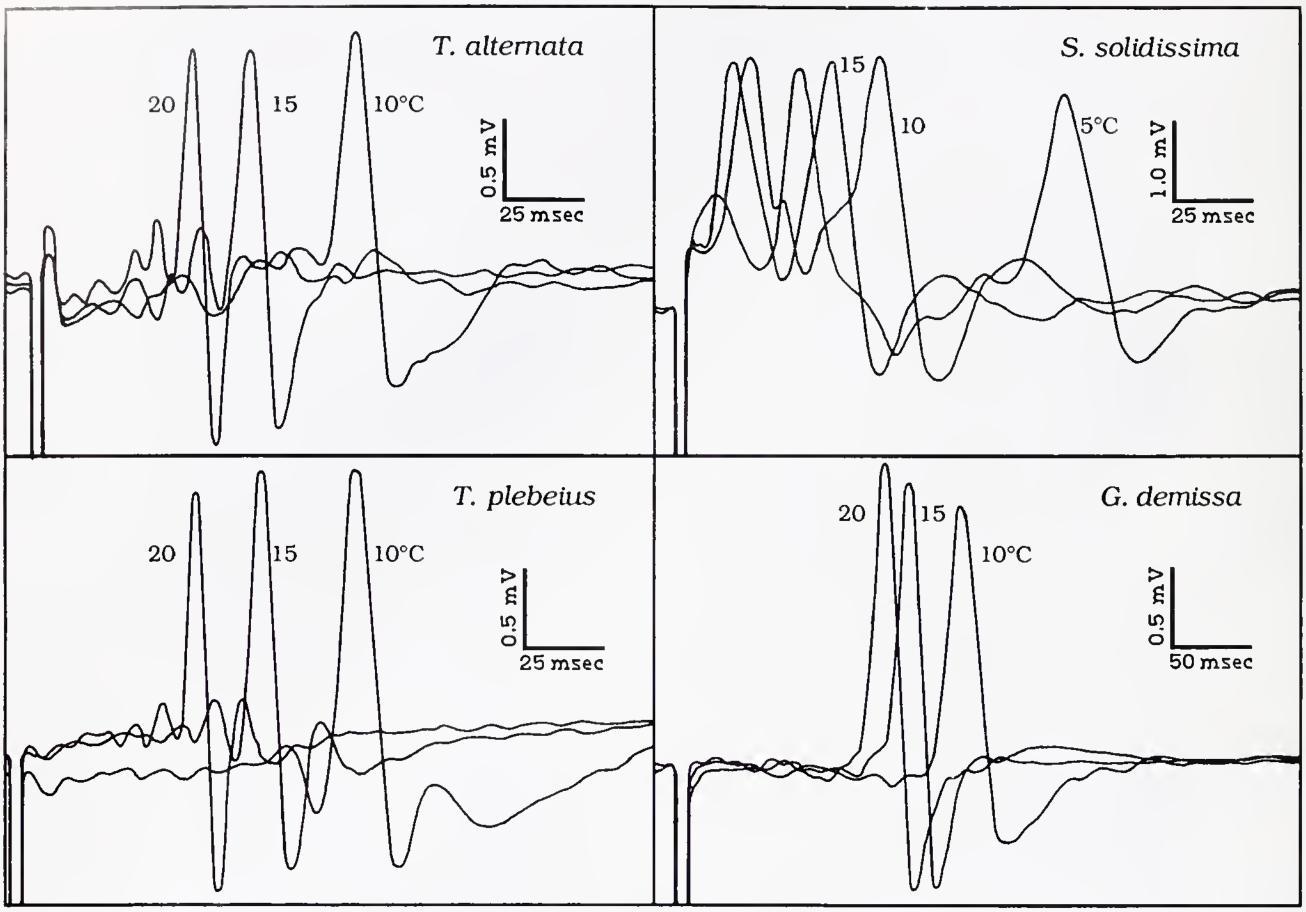

Figure 6. Externally recorded action potential traces of bivalve cerebro-visceral connectives at different temperatures. The stimulus artifact occurs on the left of each trace.

(Fig. 7). Conduction velocities corresponding to near-habitat temperatures were comparable: 21.7 ± 2.9 cm/s for *T. alternata*, 19.5 ± 3.4 cm/s for *S. solidissima*, 18.9 ± 2.4 cm/s for *T. plebeius*, and 14.7 ± 3.1 cm/s for *G. demissa*. Differences could not be attributed to the presence of neurohemoglobin ($P > 0.5$). There was no correlation between conduction velocities and conduction distances at all temperatures, indicating relatively constant conduction velocities along entire connective segments. Horridge (1958) reported 20–30 cm/s as the conduction velocity of cerebro-visceral connectives from the bivalve *Mya arenaria*. Other nerves with small diameter unmyelinated axons have similar conduction velocities: 19.4 cm/s for the garfish olfactory nerve (0.24 μm diameter axons; Ritchie and Straub, 1975) and 14.0 cm/s for the medial bundle-lateral portion of the burbot olfactory nerve (0.1–0.5 μm diameter axons; Doving and Gemne, 1965).

Increases in conduction velocity due to temperature increase were relatively similar among the connectives studied (Fig. 7). The computed temperature coefficients (Q_{10}s) showed little variation between connectives with and without neurohemoglobin. Conduction velocity Q_{10}s for other nerves with small axons are: 1.9, between 8 and 22°C, for garfish olfactory nerve (Ritchie and Staub, 1975), and 1.4, between 8 and 34°C, for burbot olfactory nerve (Doving and Gemne, 1965). Other similar Q_{10}s are 1.7, between 13 and 23°C, for giant fibers from the earthworm (Lagerspetz and Talo, 1967), and 2.2, between 5 and 30°C, for vertebrate myelinated A-fibers (Gasser, 1931).

Refractory periods corresponding to near-habitat temperatures of each species also have similar values (Fig. 8). The average absolute refractory periods are: 33.84 ± 19.36 ms for *T. alternata*, 36.65 ± 16.00 ms for *S. solidissima*, 27.04 ± 2.89 ms for *T. plebeius*, and 47.59 ± 11.56 ms for *G. demissa*. Again, the differences are not associated with the presence of neurohemoglobin ($P > 0.5$). The small axon olfactory nerves from fish exhibit similar absolute refractory periods: 28 ms for pike (Gasser, 1956) and 16 ms for burbot (Doving and Gemne, 1965). Although Q_{10}s were variable among the species

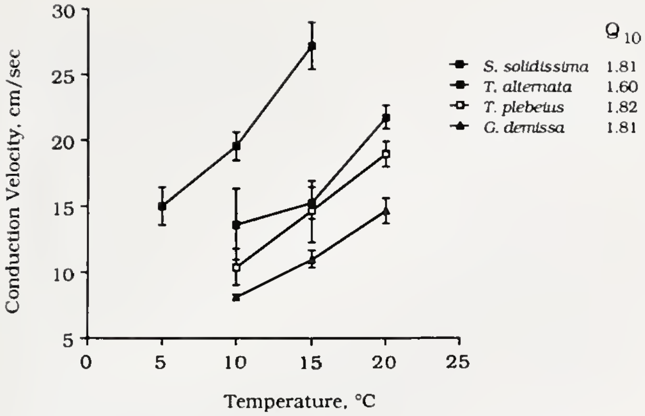

Figure 7. Conduction velocities of bivalve cerebro-visceral connectives as a function of temperature. Q_{10} values were computed over entire temperature range for each species. Data points represent the mean of 20–40 measurements (± standard error).

tested, there were no consistent differences between connectives with and without neurohemoglobin. The Q_{10}s for the absolute refractory period of vertebrate myelinated A-fibers average 5.75, between 5 and 30°C (Gasser, 1931).

Examples of threshold curves at near-habitat temperatures for the four species are presented in Figure 9. The lower stimulus strengths at each duration represent the threshold voltages required for initiating the first axons to fire and the upper values represent the maximum voltages required for recruitment of all axons. Again, consistent differences were not detectable between connectives with and without neurohemoglobin. As temperature was lowered, the curves shifted to the right as the axons became less sensitive. The changes in stimulus threshold in response to temperature were more pronounced at short duration.

Discussion

In general, the cerebro-visceral connectives with and without neurohemoglobin from the bivalve molluscs of this study are anatomically similar to each other and to homologous connectives from other bivalves (Gupta *et al.*, 1969; Willmer, 1978b). In addition, axon density, axon size distribution, and axonal membrane area per gram fresh tissue weight are similar to each other (Table II; Fig. 5) and to the unmyelinated garfish olfactory nerve, a neural preparation valued for its use in studying properties of axon membranes (Easton, 1971). The bivalve cerebro-visceral connective acts as the main communication link between the cerebropleural and visceroparietal ganglia. Consequently, the connective is free for the most part from specialized sensory and large diameter motor fibers and ganglion cells, and consists mainly of unspecialized axons (Horridge and Bullock, 1965). The garfish olfactory nerve participates mainly in relaying information from the olfactory receptors to the olfactory bulb and therefore is also composed of small unspecialized axons. In contrast, the rabbit cervical vagus (Keynes and Ritchie, 1965) and the walking leg nerves from the spider crab (Abbott *et al.*, 1958) are both multifunctional nerves and possess a broad distribution of axon sizes.

The electrophysiological characteristics of bivalve cerebro-visceral connectives are governed mainly by the axon size distribution and mean axon diameter, not by the presence or absence of neurohemoglobin. Compound action potentials are relatively simple (Fig. 6), reflecting the homogeneity of fiber size. The homogeneous fiber spectra of the pike and garfish olfactory nerves result in action potentials of similar quality (Gasser, 1956;

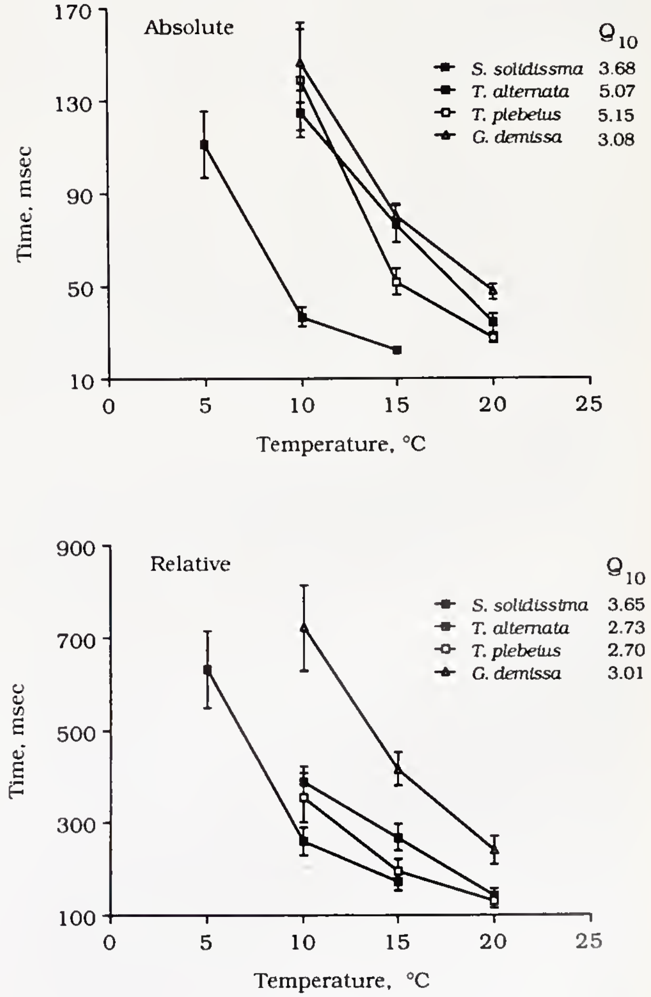

Figure 8. Refractory periods of bivalve cerebro-visceral connectives as a function of temperature. Q_{10} values were computed over entire temperature range for each species. Data points represent the mean of 15–20 measurements (± standard error).

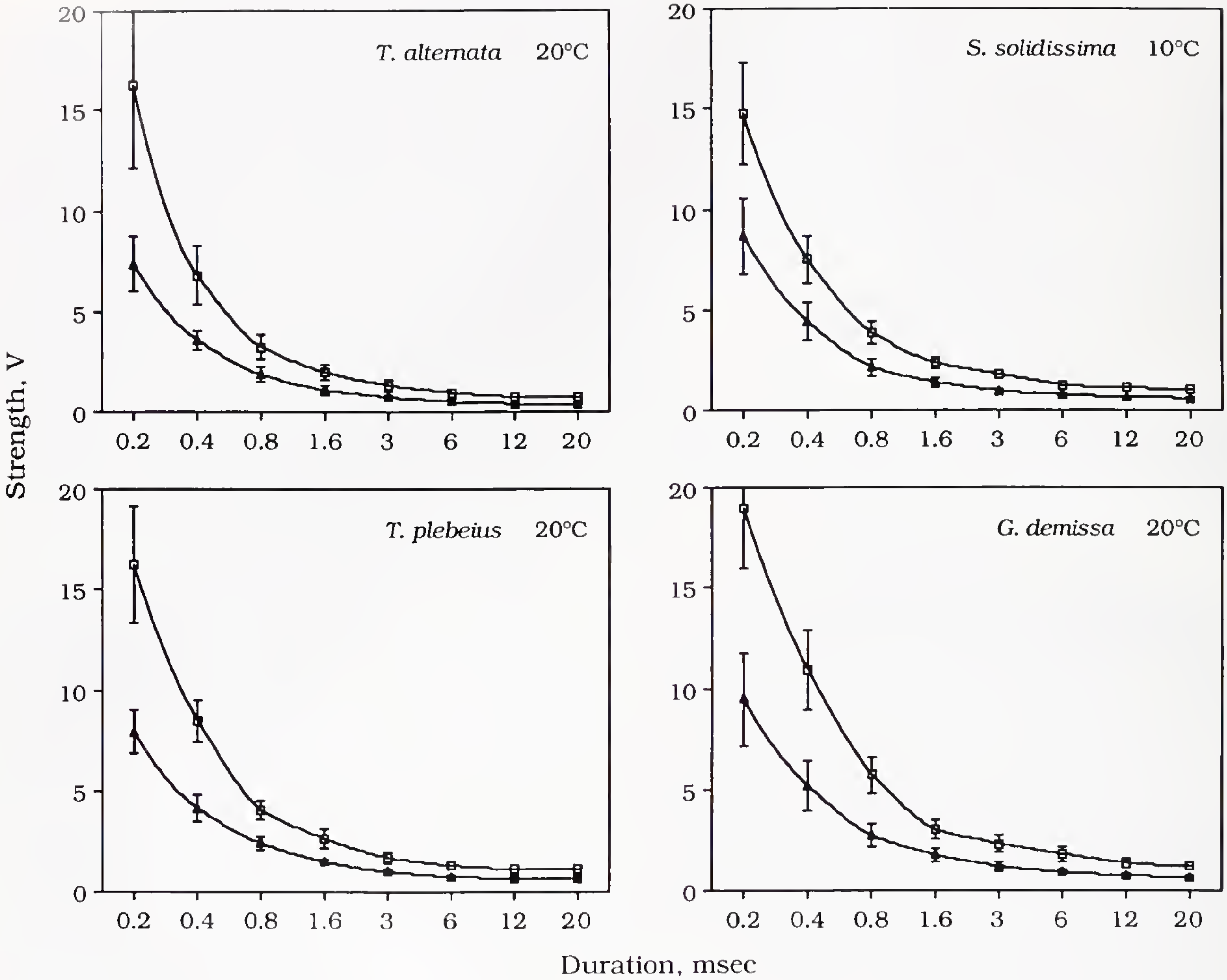

Figure 9. Strength-duration curves for bivalve cerebro-visceral connectives at near-habitat temperatures. Upper curves represent maximum recruitment of axons, lower curves represent threshold recruitment of axons. Each abscissa is scaled as a pseudo-geometric progression in order to best present the data. Data points represent the mean of 3–5 measurements (± standard error).

Ritchie and Straub, 1975). Gasser (1956) noted that this simplicity is not due merely to short conduction distances (4–20 mm in this study) because compound action potentials from the frog sciatic nerve display a complex series of peaks after 7 mm of conduction. Rather, it is due to the relatively narrow range of fiber sizes.

The small difference between the threshold stimulus voltage required for initial recruitment of axons and that required for maximum recruitment (Fig. 9) again demonstrates the constancy of axon type in cerebro-visceral connectives. Low conduction velocities indicate that axon caliber is very small (Fig. 7). Similar conduction velocities and other action potential characteristics recorded in both directions reflect the bidirectional functionality of cerebro-visceral connectives. Horridge (1958) mapped bivalve neural pathways in the whole organism and found that cerebro-visceral connectives function in bidirectional communication. These features are typical of all unmyelinated nerves with small diameter axons reported thus far (see Results for details).

Glial cells support axon function in a variety of ways such as nutrition and ion regulation (Somjen, 1975; Orkland, 1982). In bivalve cerebro-visceral connectives in which glial cells have been modified to produce hemoglobin, axon support, which may include an oxygen supply, may be more extensive than in connectives without neurohemoglobin. Based on the parameters measured under normoxic conditions at low frequency stimulation and at different temperatures, the neurohemoglobin-containing glial cells do not appear to alter action potential conduction from that exhibited by connectives without neurohemoglobin. However, glial cell participation in action potential conduction may only become evident under more stressful conditions such as low oxygen or high frequency stimulation. This is the subject of a subsequent paper (Kraus and Doeller, submitted).

In contrast to the similarity in electrophysiological characteristics described above, Kraus and Colacino (1986) reported a striking functional difference between the cerebro-visceral connectives of *T. alternata* with neurohemoglobin and *T. plebeius* without neurohemoglobin. Under anoxic conditions, the electrical excitability of connectives with neurohemoglobin lasted 20–30 minutes whereas the electrical activity of connectives without neurohemoglobin lasted only about 5 minutes (Kraus and Colacino, 1986). This functional difference (which has also been observed between the cerebro-visceral connectives of *S. solidissima* and *G. demissa:* Kraus and Doeller, submitted) may result in part from differences in the ultrastructural design of connectives with and without neurohemoglobin.

The acquisition of neurohemoglobin by glial cells to support axon function may have occurred commensurately with changes in ultrastructure that allowed maximum use of this oxygen supply. For example, smaller axon bundles caused by increased ramification of glial cell processes would have smaller mean diffusion distances between axon and glial cell. This would increase the probability that oxygen molecules released from the glia would reach the axons rather than diffuse out of the nerve. Smaller axon bundles and smaller diffusion distances are characteristic of the two cerebro-visceral connectives with neurohemoglobin (Table II). In addition, an oxygen diffusion barrier between the neurohemoglobin and the outside would tend to keep dissociated oxygen molecules within the connectives during anoxic conditions and would lengthen the time that oxygen is available for aerobic metabolism. The highly organized perineural sheath with its densely staining multilaminar structure, characteristic of the two connectives with neurohemoglobin (Fig. 2), may represent a diffusion barrier to oxygen.

To summarize, bivalve cerebro-visceral connectives with and without neurohemoglobin exhibit no significant differences in electrophysiology under normoxic conditions. Action potential conduction is influenced mainly by mean axon diameter. On the other hand, differences in ultrastructure between the two types of connectives may be related to the presence of neurohemoglobin, and oxygen delivery from glial cells to axons in neurohemoglobin-containing connectives may be aided by smaller diffusion distances and a dense perineural sheath. To understand why one type of nervous system has glial cells with neurohemoglobin and the other does not, it will be necessary to determine the functional characteristics of the neurohemoglobin, the oxygen demand of each type of cerebro-visceral connective, and the electrophysiological behavior of the connectives exposed to periods of anoxia. These will be the subjects of subsequent papers (Doeller and Kraus, 1988; Kraus and Doeller, submitted).

Acknowledgments

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A Physiological Comparison of Bivalve Mollusc Cerebro-visceral Connectives With and Without Neurohemoglobin. II. Neurohemoglobin Characteristics

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Abstract. Several bivalve mollusc species possess hemoglobin in their nervous systems whereas most species do not. The function of this neurohemoglobin was investigated *in situ* in cerebro-visceral connectives of *Tellina alternata* and *Spisula solidissima.* Both neurohemoglobins, located in glial cells, exhibit high oxygen affinities and relatively high Hill numbers. The rate of oxygen diffusion into the connective begins to fall below the consumption rate near the PO_2 at which each neurohemoglobin begins to unload oxygen, assuming the perineural sheath presents an effective barrier to oxygen diffusion. The neurohemoglobin could thus act as an oxygen store during periods of low PO_2.

Oxygen unloading from the neurohemoglobin proceeds for a considerable length of time at a constant rate. The long duration may be attributed to the geometry of the connective and to the perineural sheath, whose primary function may be to retain oxygen within the connective during anoxic conditions. The constant unloading rate may be attributed to neurohemoglobin cooperativity *in situ* because the driving force for unloading remains nearly constant at the P_{50} of each neurohemoglobin. An oxygen supply at a constant rate for an extended period of time would be useful to an animal requiring aerobic nervous function during anoxic conditions.

Introduction

A substantial amount of hemoglobin can be present in a particular tissue of one or a few species and absent in the homologous tissue of other related species. Such uncommon occurrences of hemoglobin may be supported by other adaptations (biochemical, physiological, morphological, and/or behavioral) that have secured its production. For example, only a few species of the many aquatic true bugs (Hemiptera) possess hemoglobin and it may be used for precise buoyancy regulation when the organism is submerged (Wells *et al.,* 1981). Likewise, only a small number of the multitude of meiofaunal species possess hemoglobin (Kraus and Colacino, 1984). The slowly unloading hemoglobin in *Neodasys* spp. (Gastrotricha) may provide a scuba tank of oxygen for use during excursions into anoxic zones (Colacino and Kraus, 1984). Only a few species of brittlestars (Ophiuroidae) circulate hemoglobin in their water-vascular system, possibly compensating for the lack of respiratory and reproductive bursae (Heatwole, 1981). In contrast, the terebellids (Polychaetae) possess circulating hemoglobin as a general rule, but *Lysilla alba,* which is sympatric with sanguineous species, is devoid of hemoglobin. It may compensate by more vigorous burrow ventilation (Mangum *et al.,* 1975). These examples suggest that the specific services of hemoglobin may be as diverse as the organisms themselves.

Hemoglobin is found in the nervous systems of only a few species of bivalve molluscs (Kraus *et al.,* 1988). Mollusc neurohemoglobins have been previously studied with regard to spectral and molecular properties (Strittmatter and Burch, 1963; Wittenberg *et al.,* 1965) and functional characteristics (Kennedy, 1960; Chalonzonitis *et al.,* 1965; Kraus and Colacino, 1986). Kennedy (1960) argued that hemoglobin in the nerves of *Spisula*

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solidissima was not involved in the "shadow response" of the animal. Chalonzonitis *et al.* (1965) reported that giant hemoglobin-containing ganglion cells of *Aplysia deplians* decreased their firing rate when the hemoglobin was deoxygenated. Kraus and Colacino (1986) showed that under anoxic conditions nervous activity of *Tellina alternata* cerebro-visceral connectives with neurohemoglobin lasted about 20–30 minutes, slightly longer than the duration of neurohemoglobin deoxygenation, while nervous activity of *Tagelus plebeius* cerebro-visceral connectives without neurohemoglobin lasted only a few minutes after exposure to anoxic conditions.

The bivalve cerebro-visceral connective, as the major route for communication between the cerebropleural and the visceroparietal ganglia, is relatively long and easily extracted (Kraus *et al.,* 1988). It is a useful model for investigating tissue structure and function in relation to the neurohemoglobin-bound oxygen supply. Homologous cerebro-visceral connectives from *Tellina alternata* and *Spisula solidissima* with neurohemoglobin and *Tagelus plebeius* and *Geukensia demissa* without neurohemoglobin exhibit similar electrophysiological characteristics, which are generally dictated by axon size (0.3 μm diameter axons; Kraus *et al.,* 1988). Both types of connectives possess similar morphology but several ultrastructural characteristics of the neurohemoglobin-containing connectives reflect an anatomical design that may enhance the use of oxygen stored on the neurohemoglobin (Kraus *et al.,* 1988). In this paper we show how neurohemoglobin oxygen affinity characteristics were measured *in situ* under the constraints of cellular constituents and tissue geometry. Using this method, we can determine directly how neurohemoglobin operates *in situ* and begin to understand its utility within the organism.

Materials and Methods

Specimens of *Tellina alternata* and *Spisula solidissima* were collected and maintained as previously reported and cerebro-visceral connectives were dissected as described by Kraus *et al.* (1988).

Absorption spectra

Individual pieces (approximately 5 mm long) of freshly extracted cerebro-visceral connectives from *T. alternata* and *S. solidissima* were placed in the cylindrical sample chamber of a specially designed brass gas-slide (not between membranes; *cf.* Fig. 1, Colacino and Kraus, 1984). The 0.3 ml volume sample chamber, equipped with upper and lower lateral ports for control of gas tensions, was sealed with glass lids. The slide was also equipped with internal water coils for temperature control. Experiments were performed at 20 ± 0.5°C for *T. alternata* and 15 ± 0.5°C for *S. solidissima.* The slide was positioned in a single-beam microspectrophotometer constructed from monochromator, microscope, and photomultiplier (Colacino and Kraus, 1984). A 10 × 300 μm vertical light beam was passed through the center of the horizontal connective segment so that the pathlength of light was equal to the diameter of the connective. Voltage output from the photomultiplier was monitored and recorded by a computer-aided data logger.

Absorption spectra, recorded as the cerebro-visceral connectives were exposed to flowing humidified air, N_2 or CO, were obtained by collecting the intensities of light transmitted through the connective and through a blank (a region immediately adjacent to the connective) at 5 nm wavelength intervals from 500 to 600 nm. To avoid positional artifacts caused by moving the specimen in and out of the light path, all transmitted light intensities were taken consecutively, first through the specimen and then through the blank. Relative optical densities were calculated as the difference between the optical density at each wavelength and the optical density at 600 nm, the reference wavelength. (This wavelength was chosen as reference because absorption was minimal and changed little, compared to the peak absorption wavelengths, when the neurohemoglobin changed its derivative form; Colacino and Kraus. 1984.) This method produced consistent spectra by correcting for slow random electronic drift in transmitted light intensities through both sample and blank.

Heme concentration

Volume-averaged heme concentration was determined in whole cerebro-visceral connectives of *T. alternata* and *S. solidissima.* Individual connectives in seawater were placed between a glass slide and coverslip which were separated by glass microcapillaries. Connective diameters were measured with a calibrated ocular micrometer in the eyepiece of the microspectrophotometer. A 7 μm diameter light beam was shown through the center of the connectives. Absolute optical densities of the oxygenated connectives at two peak wavelengths (540 nm and 573 nm) were calculated from the intensities of light transmitted through the sample and through a blank. The absolute optical densities of cerebro-visceral connectives without neurohemoglobin (from *Tagelus plebeius* and *Geukensia demissa;* Kraus *et al.,* 1988) were then subtracted to cancel the absorption of cellular constituents other than neurohemoglobin.

Heme concentration was also determined in individual glial cells of *T. alternata.* Connective segments were placed in depression slides, cut longitudinally, and gently teased to release the cells. Smooth spherical glial cells were easily distinguished from irregularly shaped neurons. Diameters of single glial cells were determined with the ocular micrometer. Absolute optical densities were obtained in the manner described for connective segments. Absorption due to cell membranes was assumed to be negligible.

Heme concentration was calculated with Beer-Lambert's law, using millimolar extinction coefficients for oxygenated *S. solidissima* neurohemoglobin (Strittmatter and Burch, 1963). Connective diameters or glial cell diameters were used as the light pathlength.

Molecular weight

Nearly entire nervous systems of seven *T. alternata* were carefully dissected, and portions of the nervous systems (cerebral and pedal ganglia and associated connectives) of approximately 150 *S. solidissima* were quickly excised and immersed in ice cold bivalve Ringer's (Willmer, 1978). The tissue samples were homogenized by hand in 2–3 ml cold 0.05 *M* KPO_4 buffer (pH 7.5) which contained 0.5 m*M* EDTA, and centrifuged at 10,000 × *g* for 20 min at 4°C. The pink supernatant was concentrated by N_2-pressurized ultrafiltration over an Amicon YM10 membrane (10,000 MW exclusion) at 4°C. Isolation and determination of apparent molecular sizes of the native neurohemoglobins were accomplished with fast protein liquid chromatography (FPLC, Pharmacia) using a 1 × 30 cm Superose 12 HR 10/30 column. The column was equilibrated with the same KPO_4 buffer at 30 ml/h flow rate and calibrated with 12,300–158,000 molecular weight protein standards at 20°C. The eluant was monitored at 280 nm. Absorption spectra of the collected fractions were obtained from 400 to 650 nm (Cary 17 spectrophotometer) to locate and isolate the neurohemoglobins.

Several samples of the isolated neurohemoglobins were run on denaturing sodium dodecyl sulfate (SDS) polyacrylamine gel electrophoresis (PAGE) and stained for protein with Coomassie Blue according to the methods described by Blackshear (1984). The series of protein standards (Pharmacia) ranged in molecular size from 14,400 to 94,000.

Oxygen and carbon monoxide equilibria

To measure the steady state oxygen affinity of neurohemoglobin *in situ*, short cerebro-visceral connective segments were placed in the gas-slide and arranged on the microspectrophotometer as described above. The input and output gas ports of the slide were connected to a closed circuit gas delivery system. The system consisted of a 4.6 liter glass gas mixing reservoir fitted with a polarographic oxygen sensor (with sensitivity of 0.1 mm Hg; Beckman 742 Oxygen Analyzer) and a reciprocating pump with ceramic cylinder and carbon piston (FMI). The system components were connected with 18 gauge stainless steel tubing (Small Parts, Inc.) and short couplings of thick-walled Tygon capillary tubing (Cole-Palmer). The gas reservoir was immersed in a temperature-controlled water bath. The bath also provided coolant flow to the gas-slide. All gases were humidied and the rate of gas flow through the circuit was approximately 100 ml/min.

Neurohemoglobin fractional saturation at different oxygen tensions was measured as follows. A single connective segment in the gas-slide sample chamber was exposed to flowing humidified room air. Gas flow occurred through lateral ports both above and below the specimen, thus ensuring rapid and total exposure of the specimen to the gas. An oxygenated neurohemoglobin spectrum was recorded. The slide was then flushed with humidified 99.999% N_2 to deoxygenate the neurohemoglobin. Transmitted light intensity at 560 nm, continuously monitored by the photomultiplier, was recorded with an X-Y recorder (Heathkit IR-5207). A deoxygenated neurohemoglobin spectrum was recorded when the light intensity reached an asymptote, usually in less than one hour. The neurohemoglobin was then reoxygenated in steps of 0.2–0.4 mm Hg PO_2, with absorption spectra recorded after equilibration at each PO_2. Fractional saturation was calculated from optical densities at two wavelength pairs (575 and 560 nm, 560 and 540 nm; Rossi-Fanelli and Antonini, 1958). The partial pressures of oxygen at half saturation (P_{50}) and apparent cooperativity (Hill number) for oxygen binding *in situ* were calculated from the Hill equation.

Carbon monoxide affinities of oxyneurohemoglobin and deoxyneurohemoglobin *in situ* were determined in a similar manner, except that the connectives were first exposed to air or N_2, respectively, and then to stepwise increases in PCO.

Oxygen unloading kinetics

The oxygen unloading kinetics of the neurohemoglobin *in situ* were determined with the previously described microspectrophotometer arrangement. The gas-slide containing a segment of cerebro-visceral connective was first flushed with humidified room air and [illegible]globin spectrum was recorded. The sample chamber was then flushed with humidified 99.999% N_2 and changes in light intensity at 560 nm were recorded with the X-Y recorder. The calculated time for a cylindrical air-equilibrated 0.3 ml volume sample chamber to reach 0.01 mm Hg PO_2 is approximately 2.5 s (assuming a well-mixed

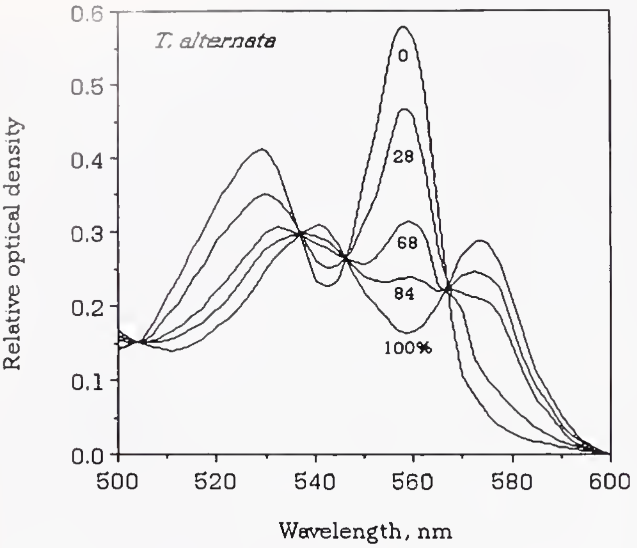

Figure 1. The oxygen equilibrium spectrogram of *Tellina alternata* cerebro-visceral connective neurohemoglobin *in situ* from 0% oxyneurohemoglobin (deoxyneurohemoglobin) to 100% oxyneurohemoglobin. Intermediate spectra were taken at 0.85, 1.55, and 2.01 mm Hg PO_2. The unusual deoxyneurohemoglobin spectrum exhibits two wavelength maxima. The spectrogram of *Spisula solidissima* neurohemoglobin *in situ*, collected at higher PO_2, is identical.

chamber), which is well below the time required for neurohemoglobin deoxygenation, approximately 30 min. At 3–5 min intervals, transmitted light intensities at 600, 575, 560, and 540 nm were recorded. A deoxyneurohemoglobin spectrum was recorded when the light intensity trace reached an asymptote. Humidified air was then reintroduced to the sample chamber and the change in light intensity at 560 nm during reoxygenation of the neurohemoglobin was recorded. Neurohemoglobin fractional saturation, calculated as before, was plotted as a function of time. The oxygen unloading rate *in situ* was calculated as the product of the slope of this curve and the heme concentration.

Oxygen dissociation using humidified CO as the deoxygenating gas was measured in a similar manner. Light intensities were collected at 570 and 535 nm, the peak wavelengths for carbon monoxide hemoglobin. In several experiments, a few crystals of dithionite were added to the seawater surrounding connective segments in a depression slide. The well was quickly sealed with a cover slip and absorption spectra were collected immediately thereafter.

Results

Spectral characteristics, heme concentration, and molecular weight

The oxyneurohemoglobin spectrum (see Fig. 1) and carbon monoxide neurohemoglobin spectrum (not shown) of *T. alternata* and *S. solidissima* cerebro-visceral connectives are similar to those of other hemoglobins. However, a distinguishing feature is the deoxyneurohemoglobin spectrum which exhibits two clear absorption maxima near 558 and 529 nm (see Fig. 1; deoxyhemoglobin typically exhibits a single maximum at approximately 555 nm). Although this spectrum is unusual, it is not unique and has been previously reported for the extracted neurohemoglobin of *S. solidissima* (Strittmatter and Burch, 1963) and the circulating plasma in the choroid rete mirabile of two fish, *Amia calva* and the bluefish (Wittenberg and Wittenberg, 1975).

The volume-averaged heme concentrations and cerebro-visceral connective diameters of *T. alternata* and *S. solidissima* are presented in Table I. Neurohemoglobin was detected spectrophotometrically only in glial cells teased from connectives and ganglia, not in ganglion cells. The heme concentration of individual *T. alternata* glial cells is 5.9 ± 1.3 m*M* (average diameter 21.2 ± 2.7 μm; n = 12), which is approximately twice the concentration in whole connectives (Table I). In accordance, glial cells represent roughly one half the cerebro-visceral connective volume because about 44% of the volume consists of axons (Kraus *et al.*, 1988), and extracellular volume is estimated at about 10% in nervous tissue (Willmer, 1978).

On the FPLC, the neurohemoglobins eluted as single dominant peaks at 28.9 ± 0.2 min (n = 3) and 29.0 ± 0.2 min (n = 3), corresponding to molecular sizes of 33,200 ± 1900 for *T. alternata* and 31,800 ± 2700 for *S. solidissima*, respectively. Elution times of neurohemoglobins and protein standards are presented in Figure 2. On denaturing gel electrophoreses, single wide bands were lo-

Table I

Characteristics of Tellina alternata *and* Spisula solidissima *cerebro-visceral connectives and neurohemoglobin* in situ

Parameter	*Tellina alternata*		*Spisula solidissima*	
Connective diameter (μm)	126	± 22 (8)*	184	± 35 (15)
Heme concentration (m*M*)	3.05	± 0.26 (18)	1.50	± 0.25 (24)
O_2 P_{50} (mm Hg)	1.3	± 0.4 (16)	2.3	± 0.3 (12)
O_2 Hill number	3.7	± 0.9 (16)	2.1	± 0.4 (12)
CO P_{50} in air (mm Hg)	0.5	± 0.2 (8)	1.3	± 0.6 (4)
CO Hill number in air	2.0	± 0.3 (8)	3.0	± 1.2 (4)
CO P_{50} in N_2 (mm Hg)	0.06	± 0.01 (9)	0.4	± 0.2 (4)
CO Hill number in N_2	3.0	± 1.0 (9)	4.1	± 1.2 (4)
Linear unloading rate with N_2 (nmol O_2 g^{-1} min^{-1})	146.2 ± 37.9 (12)		100.2 ± 24.3 (12)	

* Numbers are given as average ± standard deviation (number of repetitions).

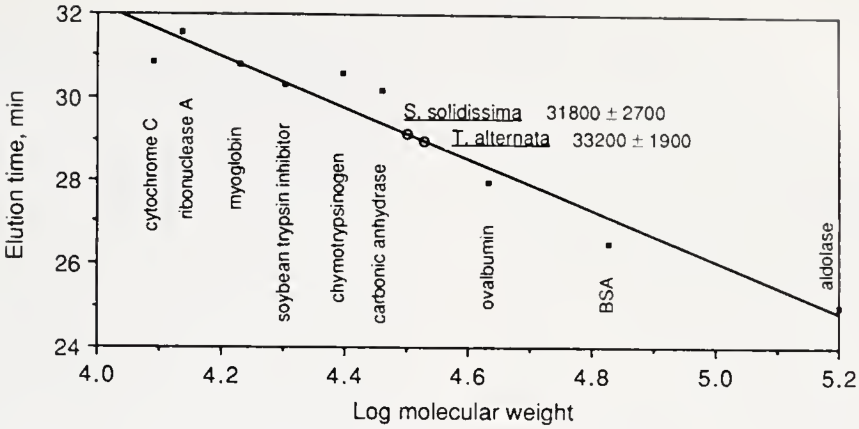

Figure 2. Molecular size determination of bivalve neurohemoglobins compared to protein standards on a Superose 12 HR 10/30 column using fast protein liquid chromatography.

cated at positions corresponding to approximately 15,000 for both neurohemoglobins, about one half the size of the native proteins. The apparent molecular sizes of both neurohemoglobins are quite similar, and each putative dimer may be composed of a single type of monomer. In general, molluscan intracellular hemoglobins in organs such as muscles, gills, and nerves are monomers or dimers with one heme per 15,000–17,000 daltons (Terwilliger and Terwilliger, 1985). Earlier reports estimated the molecular size of *S. solidissima* neurohemoglobin to be 20,000 using sedimentation coefficients (Strittmatter and Burch, 1963).

Oxygen and carbon monoxide equilibria

Characteristic absorption spectra during stepwise neurohemoglobin oxygenation are shown in Figure 1. Representative saturation curves are shown in Figure 3. Both neurohemoglobins have high oxygen affinities and exhibit high Hill numbers *in situ* (Table I). Carbon monoxide affinities of both oxygenated neurohemoglobins are in agreement with several other invertebrate hemoglobins (Table I; Wittenberg *et al.*, 1965; Bonaventura and Bonaventura, 1983). Both neurohemoglobins have a much higher affinity for CO in the absence of oxygen (Table I).

Oxygen unloading kinetics

The neurohemoglobins of *T. alternata* and *S. solidissima* required approximately 30 and 20 min, respectively, to reach 98% deoxygenation when exposed *in situ* to high purity N_2 (Fig. 4). Under these conditions, deoxygenation proceeded in a mostly linear manner that lasted approximately 70% and 50% of the total unloading duration for *T. alternata* and *S. solidissima*, respectively. The linear oxygen unloading rates are listed in Table I. In contrast, both neurohemoglobins required less than three minutes to deoxygenate when exposed *in situ* to CO. Moreover, connective segments exposed to dithionite deoxygenated in less than 2 min, the time required to place the slide on the microspectrophotometer. Dithionite creates an anoxic environment by chemically scavenging any dissolved oxygen. When fully deoxygenated and then exposed to air *in situ*, both neurohemoglobins required approximately 5 s to become fully reoxygenated.

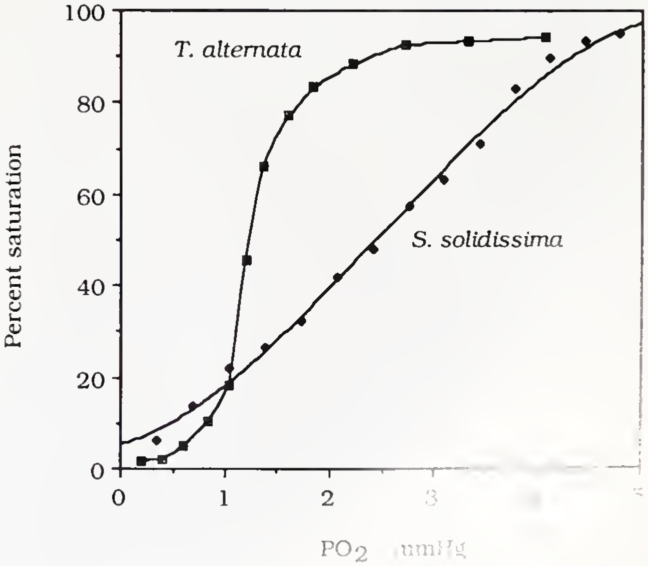

Figure 3. Steady state oxygen saturation of cerebro-visceral connective neurohemoglobins *in situ* as a function of PO_2. Both neurohemoglobins exhibit a small PO_2 range for loading and unloading, although the range for *Tellina alternata* neurohemoglobin is more narrow than the range for *Spisula solidissima* neurohemoglobin.

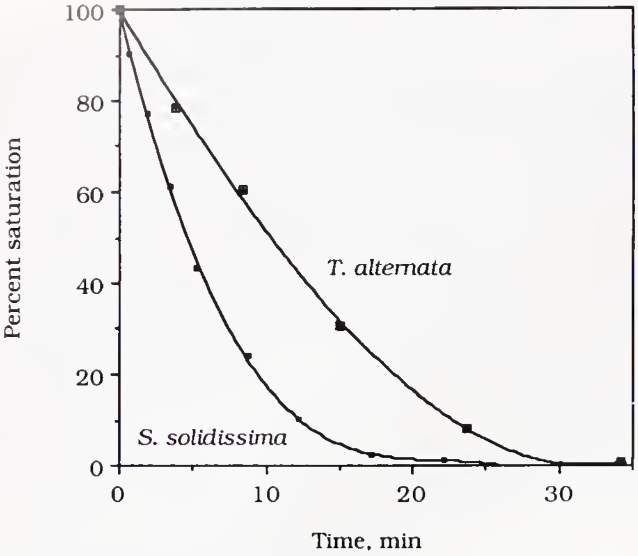

Figure 4. The change in saturation of cerebro-visceral connective neurohemoglobins *in situ* as a function of time after the start of anoxic conditions. Both curves display long duration and are linear throughout a large portion of the process.

Discussion

The red pigment in the nervous systems of *T. alternata* and *S. solidissima* is hemoglobin as evidenced by its reversible oxygenation and characteristic oxyhemoglobin and carbon monoxide hemoglobin absorption spectra. The unusual deoxyneurohemoglobin spectrum—known to be produced by pyridine hemochromagen—suggests that both the proximal and distal ligand positions of the ferrous heme iron may be ligated to nitrogenous or sulfurous groups when deoxygenated (Wittenberg and Wittenberg, 1975). This molecular arrangement may be due to reversible changes in configuration that allow intrinsic or extrinsic amino acid groups to compete for the heme distal ligand position or it may be due to partial denaturation of the protein (Wittenberg and Wittenberg, 1975). Because repeated conversion from oxyneurohemoglobin to deoxyneurohemoglobin showed no spectral signs of pigment denaturation or decrease in concentration, the hemochromagen spectrum was accepted as normal for this neurohemoglobin.

The heme concentration of both *T. alternata* and *S. solidissima* cerebro-visceral connectives is high compared to other hemoglobin-containing nerves. The heme concentration in *Aplysia depilans* ganglia, determined by a method similar to ours, was 0.04–0.1 m*M* (Chalonzonitis *et al.*, 1965). Here, the neurohemoglobin is located within giant ganglion cells and not in the glia (Chalonzonitis *et al.*, 1965). The heme concentration in the nerves of *Aplysia californica* and *Aphrodita aculeata*, determined by extraction procedures, was 0.1 and 0.7 m*M*, respectively (Wittenberg *et al.*, 1965). Neurohemoglobin concentration influences the magnitude and duration of the oxygen supply to the connectives (see below).

To understand the role of neurohemoglobin in the supply of oxygen to the axons of the cerebro-visceral connective, we consider three features of the system: (1) the geometric location of the neurohemoglobin in relation to the exterior of the connective and the oxygen-consuming axons; (2) the steady state oxygen affinity of the neurohemoglobin; and (3) the unloading kinetics of the neurohemoglobin-bound oxygen supply. In each case, the oxygen supply system of the cerebro-visceral connective of *T. alternata* operates within more restricted conditions, but the systems of both *T. alternata* and *S. solidissima* behave in a similar manner.

The bivalve cerebro-visceral connective consists of a cylindrical core of oxygen-consuming glial cells and axons, and a thin outer sheath of acellular, non-oxygen-consuming material (Kraus *et al.*, 1988). Axons transmit nervous information, glial cells offer physiological support to the axons, and the sheath provides structural support for the core. Neurohemoglobin-containing glial cells ramify extensively throughout the core to ensheath bundles of axons (Kraus *et al.*, 1988), resulting in a nearly uniform mixture of axons and entwining glial cells. Because both neurohemoglobins possess relatively low P_{50}s and high Hill numbers *in situ* (Table I) and the connectives exhibit a moderate oxygen consumption rate during activity (Kraus and Doeller, submitted), an oxyhemoglobin gradient necessary for facilitated diffusion would occur only if the connectives were exposed to a narrow and low range of ambient PO_2. The anterior portion of the cerebro-visceral connective is buried deep within the foot muscle while the posterior portion is located beneath the siphons. In addition, siphonal nerves possess neurohemoglobin with identical oxygen binding characteristics *in situ* as the connective neurohemoglobin (unpub. data), and are directly exposed to siphonal currents. Therefore, the proper PO_2 regime for facilitated diffusion from mantle fluid or from hemolymph to axons may exist at specific locations or at specific times.

Both neurohemoglobins exhibit high oxygen affinities (Table I). *T. alternata* neurohemoglobin is 95% saturated at about 3 mm Hg PO_2, computed from the Hill equation. Thus, oxygen delivery begins at a PO_2 slightly higher than 3 mm Hg. Likewise, *S. solidissima* neurohemoglobin begins to deliver oxygen at a PO_2 slightly higher than 6 mm Hg. In addition, both neurohemoglobins exhibit substantial apparent cooperativity *in situ* (Table I) and load and unload oxygen over a narrow range of PO_2 near the P_{50} of each neurohemoglobin (Fig. 3).

Neurohemoglobin could function as an oxygen store for the cerebro-visceral connective if it began to release oxygen near the PO_2 at which simple diffusion into the connective began to limit the oxygen consumption rate. This diffusion-limiting PO_2 can be calculated using the diffusion equations for a cylindrical shell (sheath) and a cylindrical core (connective) (from Hill, 1928):

$$PO_2 = [nO_2(r_o^2 - r_i^2)/(4D_c)] + [(nO_2 r_i^2)/(4D_n)]$$

where PO_2 is the external partial pressure of oxygen (atm) (assuming zero PO_2 at the center of the core), nO_2 is the specific oxygen consumption rate of the connective (nmol O_2 g^{-1} min^{-1}), r_i is the core radius (cm), r_o is the total radius (cm), and D_n and D_c are Krogh's diffusion coefficients for oxygen in muscle tissue [used here for nervous tissue, following the example of Hill (1928)] and collagen, respectively (converted to nmol O_2 cm^{-2} min^{-1} $(atm/cm)^{-1}$). For *T. alternata* cerebro-visceral connective, nO_2 is approximately 101.4 nmol O_2 g^{-1} min^{-1} during activity (Kraus and Doeller, submitted), r_i is 0.00609 cm, r_o is 0.0063 cm, and D_n is 5.98×10^{-1} and D_c is 4.91×10^{-1} nmol O_2 cm^{-2} min^{-1} $(atm/cm)^{-1}$ (Krogh, 1941). (We assume that the oxygen-consuming centers are evenly distributed throughout the connective core.) The calculated diffusion-limiting PO_2 for *T. alternata* cerebro-visceral connective is 1.3 mm Hg (1.2 mm Hg for the connective core and an additional 0.1 mm Hg for the sheath). For *S. solidissima* cerebro-visceral connective, nO_2 is approximately 96.2 nmol O_2 g^{-1} min^{-1} during activity (Kraus and Doeller, submitted), r_i is 0.0092 cm, and r_o is 0.0089 cm. The calculated diffusion-limiting PO_2 for *S. solidissima* cerebro-visceral connective is 2.8 mm Hg (2.6 mm Hg for the core and an additional 0.2 mm Hg for the sheath).

The calculated diffusion-limiting PO_2 for each connective is lower than the PO_2 at which each neurohemoglobin begins to unload oxygen (see above). If this were true *in vivo*, the diffusion rate of oxygen would not become limiting until 40–50% of the neurohemoglobin was deoxygenated. In this case, any oxygen released before diffusion-limiting conditions were reached would probably diffuse towards the oxygen-consuming axons, but would be superfluous because simple diffusion of dissolved oxygen could also supply the axons. However, if the perineural sheath was a more effective barrier to oxygen diffusion by possessing a lower diffusion coefficient than collagen or if other adjacent tissues or unstirred layers acted as additional diffusion boundaries, the diffusion-limiting PO_2 would be closer to the initial unloading PO_2 and the neurohemoglobin would be a more useful oxygen store.

The perineural sheath of both neurohemoglobin-containing cerebro-visceral connectives consists of many concentric layers of electron-dense material (*cf.* Fig. 2, Kraus *et al.*, 1988). This ultrastructure, which is unlike the ultrastructure of the more typical perineural sheath of neurohemoglobinless cerebro-visceral connectives (Kraus *et al.*, 1988), may reduce the sheath's oxygen permeability. Although a less permeable sheath would resist oxygen diffusion, the PO_2 within the tissues would probably be high enough under normoxic conditions (37–111 mm Hg, Booth and Mangum, 1978) that oxygen diffusion *into* the connective would be virtually unimpeded. However, under anoxic conditions or oxygen tensions low enough to deoxygenate the neurohemoglobin, oxygen diffusion *out of* the connective could be substantially reduced. In fact, an important function of this perineural sheath may well be to retain oxygen within the connective during anoxic conditions. Experiments are underway to determine the biochemical composition of the perineural sheaths from connectives with and without neurohemoglobin.

Two important features of the oxygen unloading reaction *in situ* are the long duration and the linearity throughout most of the unloading process (Fig. 4). Few studies have investigated the duration of tissue hemoglobin oxygen unloading *in situ* or *in vivo* where unloading behavior can be modified by cellular constituents and body geometry. The hemoglobin located in the lateral nerve cords of the nemertine *Amphiporus lactifloreus* deoxygenated in about 3 min when the animal was sealed between a glass slide and cover slip and denied access to oxygen (Varndell, 1980). In contrast, the hemoglobin of the microscopic vermiform gastrotrich *Neodasys* spp. (less than 50 μm in diameter) required nearly one hour to deoxygenate when the animal was placed between two 6 μm Teflon membranes and exposed to 99.999% N_2 (Colacino and Kraus, 1984). Similarly, the hemoglobin in the anterior hypodermal cells of the nematode *Mermis nigrescens* remained oxygenated for more than two hours when perfused with Ringer's solution equilibrated with high purity N_2 (Burr and Harosi, 1985).

In general, oxygen unloading from a hemoglobin-containing tissue can be limited either by the molecular dissociation rate of the hemoglobin (unloading is reaction-limited) or by the diffusion rate out of the tissue (unloading is diffusion-limited) (Colacino *et al.* 1987). An oxygen molecule once dissociated can be removed from the tissue via metabolic consumption or diffusion to the exterior, or it can reassociate one or more times to other deoxyhemoglobin molecules. Once the oxygen molecule reaches the tissue exterior, it can be removed from the boundary layer by diffusion or convection. With little or no diffusion resistance within or surrounding the tissue, unloading time will be controlled by the molecular dissociation rate. In this case, long unloading times will be displayed by hemoglobins with low dissociation rate constants. For example, the hemoglobin of *Ascaris lumbricoides* would require more than 15 min to deoxygenate, given its rate constant (0.004 s^{-1}) determined by stopped-flow techniques (Gibson and Smith, 1965).

The possibility that neurohemoglobin may possess low molecular dissociation rates was investigated by using CO or dithionite to deoxygenate the neurohemoglobin. Because neurohemoglobin exhibits a much higher affinity for CO than oxygen (Table I), CO preferentially binds to any heme iron exposed by oxygen dissociation. Oxygen reassociation is thus prevented and the rate of oxygen dissociation *in situ* can be approached, limited mostly by the rate of CO diffusion into the connective. Under these conditions, deoxygenation times were greatly reduced (see Fig. 5A, curve c). Dithionite, which scavenges oxygen, also greatly reduced deoxygenation times. The neurohemoglobin, therefore, probably does not have an exceptionally low molecular dissociation rate, and oxygen unloading from the connective is not reaction-limited.

The unloading time of a diffusion-limited reaction is controlled by the combined effect of the diffusion rate of oxygen out of the region plus the number of dissociations and reassociations that take place before oxygen reaches the exterior (Colacino *et al.*, 1987). The diffusion rate can be limited by a specific diffusion boundary located between the hemoglobin and the exterior. Moreover, the reassociation of oxygen to deoxyhemoglobin is more likely when hemoglobin concentration is high and when oxygen permeability of the diffusion boundary is low. These factors will lengthen oxygen unloading times. Preliminary data indicate that unloading times are shorter when neurohemoglobin concentration is effectively lowered by partial inactivation with CO.

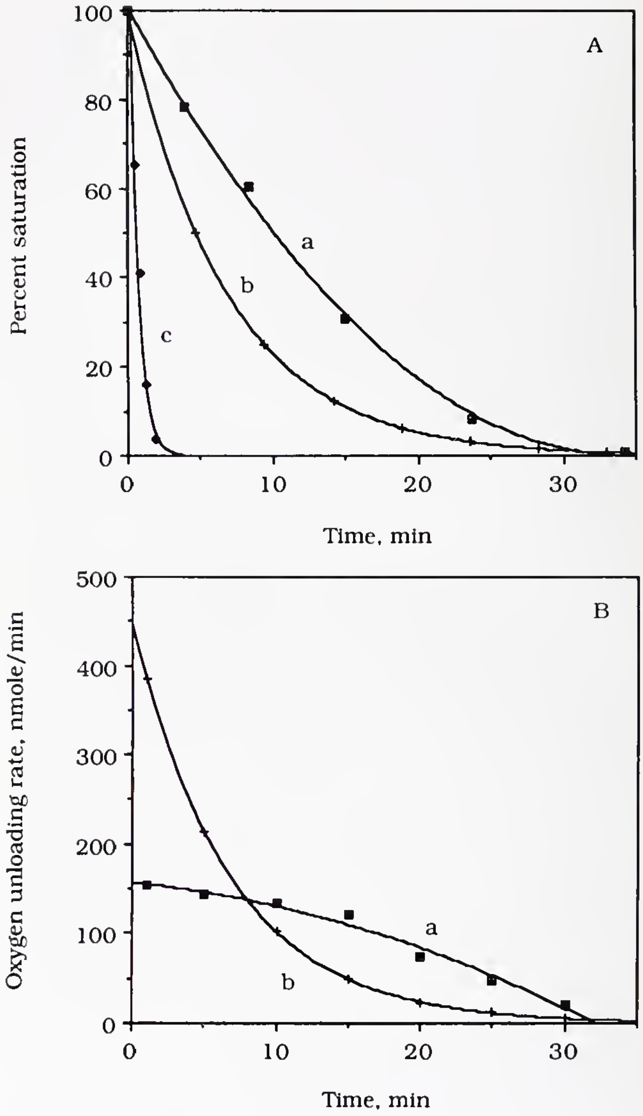

Figure 5. A. (a) The oxygen unloading curve of *Tellina alternata* cerebro-visceral connective neurohemoglobin *in situ*. (b) A hypothetical exponential unloading curve of the same duration as curve a. (c) The oxygen unloading curve of *Tellina alternata* neurohemoglobin *in situ* with CO as the deoxygenating gas. B. (a) The derivative of curve a in A. (b) The derivative of curve b in A. (See text for discussion.)

The time for oxygen unloading can be estimated from the quantity of neurohemoglobin-bound oxygen and the diffusion rate across the sheath, assuming the sheath represents the principle diffusion barrier. The steady state rate of diffusion (neglecting metabolism) out of a 1 cm length of cylindrical shell like a sheath, Q (nmol O_2 cm^{-1} min^{-1}), can be calculated using

$$Q = (2\pi D_c dPO_2)/\ln(r_o/r_i)$$

where D_c is Krogh's diffusion coefficient for oxygen in collagen (4.91×10^{-1} nmol O_2 cm^{-2} min^{-1} $(atm/cm)^{-1}$), dPO_2 is the partial pressure difference across the sheath (atm), and r_o and r_i are the outside and inside radii (cm), respectively, of the cylinder (Jacobs, 1967). This steady state equation was selected because the neurohemoglobin unloading rate is nearly constant throughout most of its duration, although more so in *T. alternata* than in *S. solidissima* (Fig. 4). A nearly constant unloading rate implies that neither the partial pressure gradient nor the diffusion distance is changing within the core of the connective, thus we assume that the core is a well-mixed solution at constant PO_2 near the P_{50} (see below; Honig *et al.*, 1986). For an average *T. alternata* connective, dPO_2 is 1.3 mm Hg (Table I) or 1.71×10^{-3} atm, and r_o and r_i are defined previously. The calculated steady state rate of diffusion, Q, is 1.6×10^{-1} nmol O_2 min^{-1} cm^{-1}. The quantity of oxygen in this 1 cm segment is 3.8×10^{-1}

nmol O_2, computed from the cylinder volume and the average heme concentration (Table I). Dissolved oxygen is negligible at this low PO_2. The unloading time, computed by dividing the quantity of oxygen by the rate Q, is 2.4 min. The unloading time computed for an average *S. solidissima* connective segment is 1.4 min, using 4.0×10^{-1} nmol O_2 per 1 cm segment and 2.3 mm Hg dPO_2 to give a Q of 2.8×10^{-1} nmol O_2 min^{-1} cm^{-1}. Both calculated times are clearly shorter than the observed 20 min and 10 min, respectively, for the approximate linear portion of the unloading process (Fig. 4), but again, they would be longer if the oxygen permeability of the sheath were lower or if dPO_2 was smaller.

The second unusual feature of the oxygen unloading reaction *in situ* is its linearity during a large portion of the process. The apparently cooperative nature of neurohemoglobin *in situ* may be responsible for this phenomenon. Because PO_2 in the connective is effectively held within a narrow range near the P_{50} (Fig. 3), the driving force for unloading is nearly constant. In this study both native neurohemoglobins have been isolated only as oxygenated dimers, but further subunit interaction is possible. Cooperativity results from subunit interaction which for some hemoglobins is enhanced by aggregation upon deoxygenation (Chiancone *et al.*, 1981; Tam and Riggs, 1984; Wells *et al.*, 1984).

To illustrate the physiological significance of the unusual oxygen unloading features (Fig. 5A), the neurohemoglobin dissociation reaction *in situ* (curve a) and an hypothetical first order dissociation reaction (curve b) of equal duration are presented for comparative purposes. The derivatives of both reactions plotted against time (Fig. 5B) indicate how changes in the oxygen unloading rate with time differ between the two types of reactions. The neurohemoglobin unloading rate (Fig. 5B, curve a) is nearly constant and independent of oxyhemoglobin concentration throughout most of the unloading process. In contrast, the unloading rate of the exponential reaction (Fig. 5B, curve b) is proportional to oxyhemoglobin concentration and thus declines with time.

We argue that the geometric design of the neurohemoglobin-containing cerebro-visceral connective and the oxygen affinity characteristics of the neurohemoglobin are concurrently responsible for the magnitude and duration of the neurohemoglobin-released oxygen supply. An oxygen supply at a constant rate for an extended period of time would clearly be useful to an animal requiring aerobic nervous function during anoxic conditions, and both *T. alternata* and *S. solidissima* often burrow into anoxic muds. In a subsequent paper, we investigate the system's efficiency, including the amount of neurohemoglobin-released oxygen actually consumed by the connective and how well the connective maintains activity under anoxic conditions (Kraus and Doeller, submitted).

Acknowledgments

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The Relationship Between Subunit Composition and O_2 Binding of Blue Crab Hemocyanin

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Abstract. Hemocyanin-O_2 affinity differs in estuarine and seaside populations of the blue crab *Callinectes sapidus.* The difference, which is adaptive, is accompanied by different proportions of the six subunits that make up the native hemocyanin polymers. When the hemocyanins are dissociated and separated by alkaline electrophoresis, six subunits can be resolved in most estuarine individuals but two of the six are either present in low concentrations in or absent from most seaside individuals. A third subunit is also variable but the variation is not clearly correlated with locality. O_2 binding measurements of Hcs with the two major wild phenotypes but collected at the same locality reproduce the difference between the natural populations. Measurements on several intermediate phenotypes suggest that the variation of one of the subunits is more important physiologically than variation of the other two.

Introduction

Mason *et al.* (1983) found that estuarine and seaside populations of the blue crab *Callinectes sapidus* Rathbun, which are separated by about 200 km, have different intrinsic O_2 affinities of their hemocyanins (Hcs). The differences ensure adequate O_2 transport in each locality despite large changes in blood levels of several allosteric effectors (Mangum, 1986). After eight days estuarine animals transferred to high salinity had a HcO_2 affinity indistinguishable from the seaside phenotype, and seaside animals transferred to low salinity exhibited a shift in HcO_2 affinity towards the estuarine phenotype. Cooperativity did not change and the response of the Bohr shift was not examined.

Although Mason *et al.* (1983) did not reach a firm conclusion concerning the factor responsible for the acclimation, they did report a difference in the Hc subunit composition of pooled samples taken from six to seven experimental animals representing each locality. While six subunits were clearly present in the estuarine animals immediately after collection and still acclimated to estuarine conditions, only four were detected in seaside animals immediately after collection and still acclimated to seaside conditions. Perhaps most important, a shift in Hc subunit composition towards the seaside phenotype accompanied the shift in O_2 affinity following transfer of estuarine animals to high salinity. (The sample from the seaside group transferred to low salinity was unavailable for analysis.) Hc concentration rose in animals transferred from high to low salinity and fell in animals transferred from low to high salinity. This is consistent with a hypothesis of net synthesis of selected subunits at low salinity and net degradation at high salinity. Mason *et al.* (1983) tentatively advanced the hypothesis that the acclimation might result from rearrangement of the proportions of functionally distinct subunits, a mechanism long the subject of conjecture (*e.g.*, Bonaventura and Wood, 1980).

A preliminary examination of the phenotypes of 51 individuals from each of the two localities revealed variability of a third subunit which is not clearly correlated with salinity. A detailed examination of the distribution of the various phenotypes is now under way [illegible] *al.*, 1985).

To determine the subunit composition [illegible] individual crabs as well as O_2 affinity, we attempted to collect larger volumes of blood while repeating the transfer experiment. Our attempts met with limited success due to the greater mortality that accompanied heavier bleeding. Therefore, we performed somewhat less direct but also less invasive experiments designed to test the hypothesis that the Hc phenotypes observed in estuarine

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and seaside crabs are responsible for the differences in HcO$_2$ affinity. Because HcO$_2$ affinity also changes with season (Mauro and Mangum, 1982), we performed our experiments during July—the same month in which previous experiments were performed (Mason *et al.*, 1983).

Materials and Methods

We either captured or purchased crabs from local watermen whose pots had been placed at known locations. Water salinity was determined, by conductivity (Yellow Springs Instrument Co. Model 33 salinometer), in both cases.

As in the previous investigation (Mason *et al.*, 1983), a transfer experiment was designed as paired observations on the same individuals before and after a salinity change. Blood was taken from each individual before and after the transfer. We intended to save one aliquot for electrophoresis and use the other for O$_2$ binding. Poor survival dictated otherwise and we used all of the material available to ascertain our earlier finding of a shift in HcO$_2$ affinity.

As an alternative to holding previously bled animals in the laboratory at the time of year when mortality is already great, we observed different phenotypes at the estuarine (York River) and seaside (Wachapreague) habitats. We first sampled large numbers of animals, then identified the phenotypes electrophoretically, and finally performed O$_2$ binding measurements on them.

HcO$_2$ equilibrium measurements were made using the cell respiration method (Mangum and Lykkeboe, 1979). After clotting the samples were homogenized in a tissue grinder, centrifuged, and dialyzed overnight against a saline.

Regression lines were fit to the data describing log P$_{50}$ as a function of pH and the 95% confidence intervals around the lines and their slopes were compared for overlap. Values for P$_{50}$ at a single pH and those for cooperativity were analyzed according to Student's *t*-test.

Hcs were dissociated by dialysis against 0.05 *M* Tris HCl (pH 8.9) for 12–16 h. In the early experiments polyacrylamide gel electrophoresis was performed as indicated by Mason *et al.* (1983). Later the gels were prepared according to the method of Hames and Rickwood (1985), which gave better separations. The two protocols differ only in the concentrations of buffer, crosslinker, and TMED (N,N,N',N'-tetramethylethylenediamine). All of our gels (0.75 mm × 16 cm) consisted of a 3% stacking gel over a 12.5% resolving gel. A discontinuous buffer system was used, with Tris-glycine buffer (pH 8.1) in the upper chamber and Tris-HCl buffer (pH 8.9) in the lower chamber. Best separation was obtained when the electrophoresis proceeded for 8–12 h at constant current (15–18 mA). The gels were stained for 40 min with 0.1% Coomassie blue (G-250) in 25% trichloroacetic acid and destained with a mixture of acetic acid and methyl alcohol.

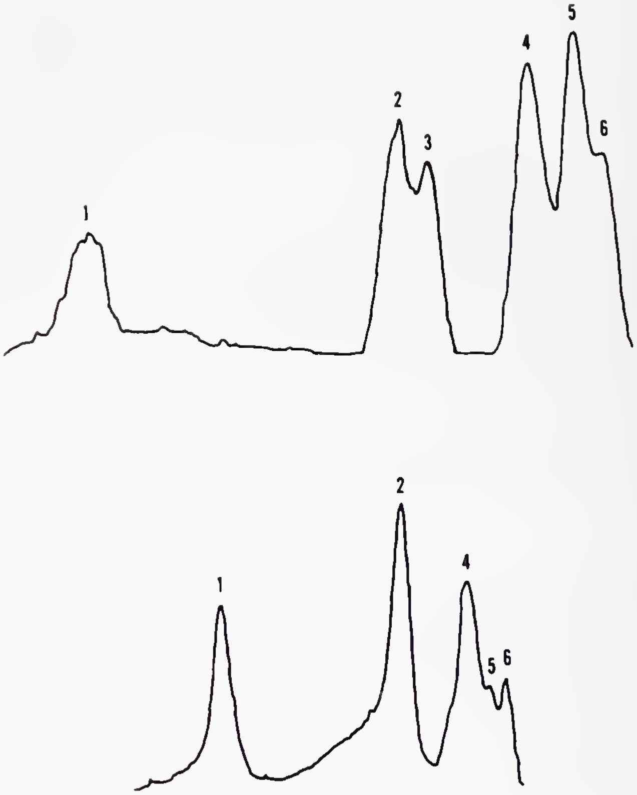

Figure 1. Densitometer scans of Hc wild phenotypes. Top: estuarine population (10 cm/min scan); bottom: seaside population (2 cm/min scan).

Results

Here and in the Discussion below we designate the electrophoretic phenotypes as follows. Maximal concentrations of all six subunits was the predominant phenotype in our preliminary sample from the upper York River estuary of Virginia, where the typical salinity range is 3–16‰. This "estuarine wild phenotype" is symbolized as HHHHHH (Fig. 1). Minimal concentrations or absence of subunits 3 and 5, was the predominant pattern at Wachapreague, Virginia, where the salinity normally ranges from 29 to 33‰. This "seaside wild phenotype" is symbolized as HHLHLH (Fig. 1). Additional phenotypes examined here include minimal concentrations of subunits 3 and 6 together (HHLHHL), subunit 3 alone (HHLHHH), and subunit 6 alone (HHHHHL). Still another phenotype (HHLHLL) has been recovered on occasion, but not in the samples examined here.

Each of three attempts to collect large volumes of blood while repeating the transfer experiment met with

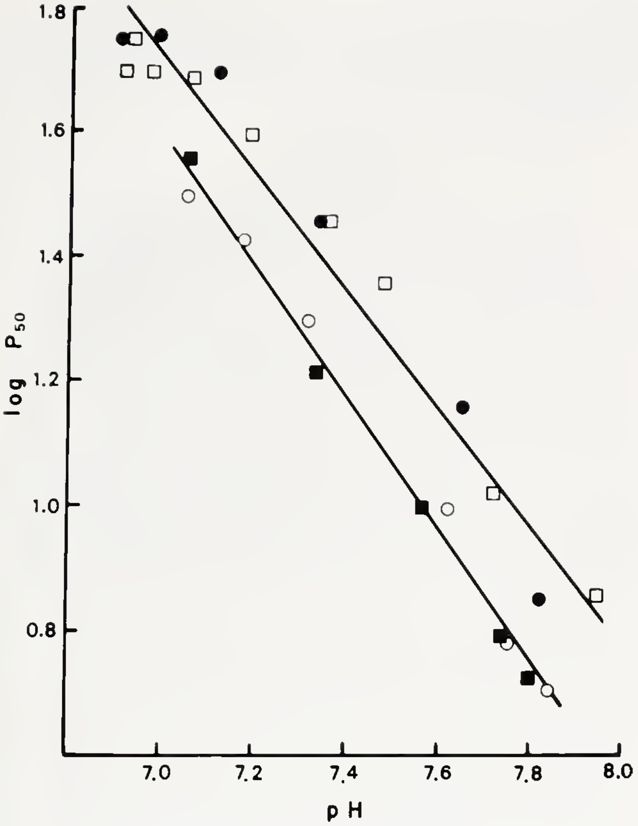

Figure 2. Relationship between pH and P_{50} of Hcs dialyzed against 0.05 *M* Tris maleate buffered seawater (32‰). 25°C. (●) Blood from estuarine crab taken immediately after collection. (○) Blood from same crab held 10 days at 32‰. (■) Blood from seaside crab immediately after collection and (□) blood from same crab after 11 days at 5‰.

little success due to mortality, a common problem at summer temperatures. In the first two attempts all 14 animals in each group were dead by the end of 5 days. The O_2 binding properties of the bloods of several of these animals examined had not changed significantly during the acclimation period.

In the third attempt a single individual (of 21) in each group survived. Of those animals transferred from 32 to 5‰, one individual survived for 11 days, and of those transferred from 8 to 32‰, one individual survived for 10 days. Daily feeding (shrimp) and/or a two-step transfer did not enhance survival. The O_2 binding measurements made at physiological pH on samples from the two populations (Fig. 2) agree with the trends reported earlier, as do those made 10–11 days following transfer to the alternative salinity. We should note that the absolute values in these data are slightly different, most likely because the unavailability of chemicals at our seaside laboratory made it necessary to dialyze the samples against seawater rather than a strictly physiological saline. The difference between the two populations is also slightly greater than observed before or since, possibly due to different inorganic ion sensitivities of the two phenotypes. Regardless, HcO_2 affinity in the sole survivor of the transfer in either direction is indistinguishable from that of the alternative population. The present results show no difference in the Bohr shift.

We did not regard results from a single animal as an adequate test, so we adopted a new approach. Forty-five animals were caught at various locations in the York River estuary and its tributaries; animals with the two wild phenotypes were identified. Large blood samples were taken without regard for survival, and divided. One aliquot was electrophoresed and, simultaneously, another aliquot was used for O_2 binding. Due to the large number of samples analyzed and the small number with the seaside phenotype recovered, the observations were confined to physiological pH. The results are shown in Table I. There is clearly a difference ($P < .001$) between the estuarine (collected at 5‰) wild phenotype and the seaside wild phenotype, which was collected on this occasion at low salinity (2‰).

In our final experiment we sampled the seaside population (n = 55) which, at least occasionally, has proven to be more diverse than estuarine populations and, since each chain appears to vary independently of the others, presented the possibility of yielding intermediate as well as both wild phenotypes. On this occasion we identified the phenotypes prior to the O_2 binding measurements so that we could discard unneeded duplicates and make more extensive observations on any one. The measurements, which were completed within 5 days of sample collection, indicate that at physiological pH the difference in the P_{50} values (antilog) for the two wild phenotypes, both collected at the seaside locality (32‰), is 30–35% (Fig. 3). This is the same as the difference between the two wild phenotypes collected at their respective natural localities (Mason *et al.*, 1983).

This set of observations appears to show that the absolute difference between the two wild type phenotypes diminishes when P_{50} becomes very small above pH 7.8, a range not examined earlier. However, the data for intermediate phenotypes (Fig. 4) suggest that this tendency may not be real. More importantly, in the 7.0–7.8 pH range data for the intermediate phenotypes lie between

Table I

O_2 binding of Hc phenotypes found in sample of estuarine blue crabs[1]

Phenotype	pH	P_{50}	n_{50}
HHHHHH	7.50	21.8 ± 0.7 (6)	3.79 ± 0.34 (6)
HHLHLH	7.53	15.2 ± 0.3 (4)	3.16 ± 0.25 (4)

[1] Dialyzed against buffered saline described in legend of Figure 2. 25°C. Mean ± S.E. (N).

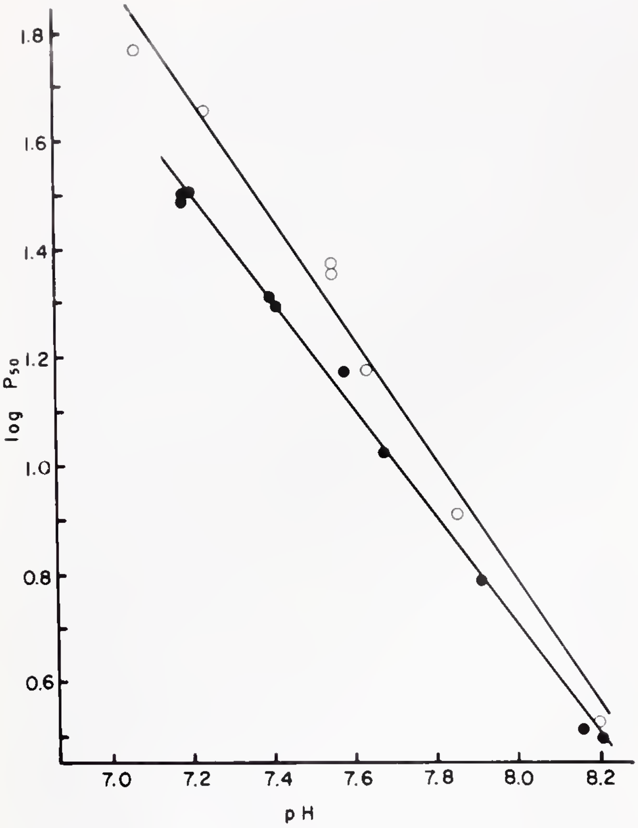

Figure 3. Relationship between pH and P_{50} of samples taken from one seaside crab with HHHHHH phenotype (○) and three seaside crabs with HHLHLH phenotype (●). Dialyzed against 0.05 *M* Tris maleate buffered saline containing 368 m*M* NaCl, 12 m*M* KCl, 18 m*M* $CaCl_2$, 21 m*M* Na_2SO_4, 17 m*M* $MgCl_2$, and 3 m*M* $NaHCO_3$ (Mason *et al.*, 1983). 25°C. The lines fitted by regression analysis differ at $P < .001$.

those for the two wild phenotypes (Fig. 4). When subunit 6 alone drops from high to low concentrations (HHHHHH to HHHHHL), HcO_2 affinity also decreases; the regression line describing the data differs from that for the estuarine wild type throughout the pH range examined. It also differs from the seaside wild phenotype (HHLHLH) throughout the entire pH range. However, when band 3 alone is low (HHLHHH), HcO_2 affinity differs very little from that of the seaside wild phenotype (HHLHLH). The differences are significant only at the pH extremes (7.0–7.2 and 8.2). Moreover, the HHLHHH phenotype differs from HHHHHH (estuarine wild phenotype) throughout the entire pH range. A decrease in concentration of subunit 6 as well (HHLHHL) has little or no effect: the data for this phenotype differ from those for HHLHHH only slightly and only at pH 7.0–7.4. They differ from HHHHHH throughout the pH range. The differences are summarized in Table II and the O_2 affinities at physiological pH are specified in Table III. Like Mason *et al.* (1983), we found no differences in cooperativity between any of the phenotypes (P = .15–.82); the magnitude of the Bohr shift (described by slopes of the regression lines) also failed to differ significantly ($P > .05$).

Discussion

The role of subunit heterogeneity in the assembly of native Hc polymers is rapidly being elucidated (Markl, 1986; Markl *et al.*, 1986; Stöcker *et al.*, 1986). Using mixtures of purified subunits Stöcker *et al.* (1986) found that the maximum amount of assembly to the native dodecamers of *C. sapidus* Hc is achieved only when all six subunits are present in approximately physiological ratios. To clarify this comparison we should note that Stöcker *et al.*'s (1986) order of numbering the bands is the opposite of ours, viz. it progresses from anodic to cathodic rather than the other way around as practiced here and also by Mason *et al.* (1983) and Johnson *et al.*

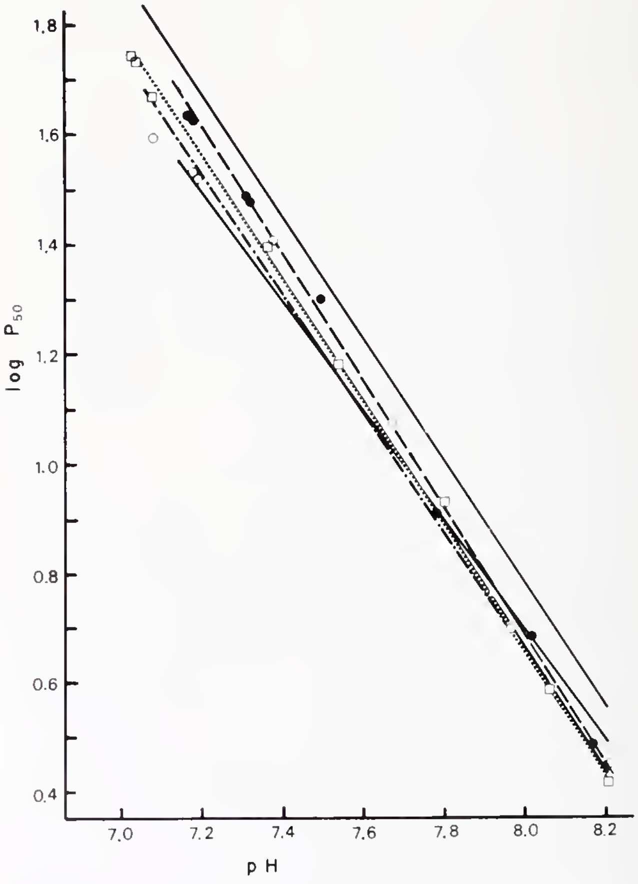

Figure 4. Relationship between pH and P_{50} of samples from two seaside crabs with HHHHHL phenotype (●, dashed regression line), three with HHLHHH phenotype (○, dashes and dots), and two with HHLHHL phenotype (□, dotted line). Dialyzed against buffered saline described in legend of Figure 2. 25°C. The solid lines reproduced from Figure 2 describe the two wild type phenotypes.

Table II

Differences in HcO_2 affinities of the five phenotypes recovered in Wachapreague in 1986

	HHLHLH	HHLHHL	HHLHHH	HHHHHL	HHHHHH
HHLHLH	—	*	*	**	**
HHLHHL	*	—	*	**	**
HHLHHH	*	*	—	*	**
HHHHHL	*	**	*	—	**
HHHHHH	**	**	**	**	—

** Significant (no overlap of 95% confidence intervals around regression lines in Figs. 2 and 3) throughout pH range examined.
* Significant only in limited pH range. See text for details.

(1984). We should also note that the gel scans shown by Stöcker *et al.* (1986) appear to us to represent the estuarine wild phenotype in that all six bands are easily detectable, although comparison is somewhat difficult since they used the crossed immunoelectrophoresis technique.

Based on a combination of characteristics derived from electrophoretic mobility, the immunological response, antigenic sufficiency, and the role in protein assembly, Markl (1986) and Markl *et al.* (1986) devised a scheme of classifying the arthropod Hc subunits. In their terminology the three variable subunits in *C. sapidus* are one alpha prime (our subunit 3), one alpha (subunit 5), and one gamma (subunit 6) chain and the invariant (at least thus far) subunits are two betas (1 and 4) and one alpha (2).

Native blood in this species contains a mixture of hexamers and dodecamers in a ratio reported to be anywhere from 1:4 (Hamlin and Fish, 1977; Johnson *et al.*, 1984) to as much as 1:1, possibly due to genuine physiological variation (Herskovits *et al.*, 1981). Any one of the three kinds of chains is competent to form hexamers but the maximum yield is obtained when small amounts of gamma chains are added to purified preparations of alpha chains (Stöcker *et al.*, 1986). The second step in assembly of dodecamers, a physiologically important process (Snyder and Mangum, 1982; Mangum, 1986), requires the alpha prime chains, which are believed to serve as the interhexamer bridgers. Although one of the two alpha chains (subunit 5) in *C. sapidus* is frequently low or absent in seaside crabs, the apparent invariance of a second alpha chain (subunit 2) may ensure the formation of at least hexamers. The variability of alpha prime chains, believed to play an integral role in further assembly, however, raises the question of whether a changing ratio of hexamers to dodecamers is responsible for the differences in HcO_2 affinity observed here.

Table III

O_2 affinities at pH 7.6 and 25°C of Hc phenotypes collected from seaside population

Phenotype	P_{50}	95% confidence interval
HHHHHH	16.7	16.4–17.1
HHHHHL	14.3	14.2–14.3
HHLHHL	12.9	12.8–12.9
HHLHHH	12.5	12.4–12.5
HHLHLH	12.6	12.5–12.6

Data from Figures 3 and 4.

Johnson *et al.* (1984) reported that the hexameric fraction found in the blood of *C. sapidus* lacks subunit 5, one of the alpha variables, and subunit 6, the gamma variable. Since subunits 2 and 3 were not separated it is not clear whether Johnson *et al.*'s (1984) scans of dodecamers resemble those of our HHLHHH, a not especially common phenotype, or HHHHHH, the estuarine wild phenotype. Johnson *et al.* (1984) mentioned that the O_2 affinities of hexamers and dodecamers are similar, although no data are shown. According to their scans hexamers should have either a HHHHLL phenotype, which we have never recovered, or a HHLHLL phenotype, which is very rare and for which we have no O_2 binding data. However, if, as suggested above, the variability of subunit 5 is of little or no physiological importance, then the O_2 binding of HHHHLL should resemble that of HHHHHL, which clearly differs from the two wild phenotypes although its relationship to the rare HHLHLL remains unknown.

Herskovits *et al.* (1981) implied that the observed range in the ratio of hexamers to dodecamers was correlated with season. Due to the potential importance in relation to the present findings and also to the seasonal difference in O_2 binding (Mauro and Mangum, 1982), a thorough investigation of the O_2 binding behavior of hexameric and dodecameric fractions would seem to be in order.

In any event, the present results confirm the earlier finding of differences in the respiratory properties of the HcO_2 transport system within the species *Callinectes sapidus*. Due to the paucity of the data, they less strongly support the earlier inference that the adaptation is acclimatory and therefore non-genetic in origin. Indeed, none of our work, past or present, even pertains to the genetic status of the variability of subunit 6. But our present findings convincingly argue for a functional as well as a structural role of the various subunits, and a functional importance of phenotypic variability.

Acknowledgment

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Gas Supersaturation Thresholds for Bubble Formation in and Damage to Sea Urchin Embryos

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Abstract. Eggs and early embryonic stages of the sea urchin *Lytechinus pictus* were subjected to hyperbaric gas pressures and rapid decompression. All stages showed a remarkable tolerance to gas supersaturations. No damage or internal bubbles were apparent in the eggs after decompression from less than 240 atm nitrogen or 209 atm argon. This indicates (1) a greater resistance to bubble formation than occurs in other invertebrates and vertebrates and (2) a lack of nucleation sites, such as hydrophobic interfaces in contact with the intracellular water. These thresholds decreased gradually to 170 atm and 148 atm, respectively, for 80-h-old plutei. Gas supersaturations above the threshold values often led to formation of internal bubbles, most frequently observed in the eggs. Slow decompression experiments usually had little effect on the organisms, showing that gas supersaturations were the cause of the damage rather than the hydrostatic pressures, the gases *per se,* or the hyperbaric conditions inherent in the procedures.

Introduction

In studies of bubble formation in cells subjected to hyperbaric gas pressures and subsequent decompression, it has been found that unicellular organisms such as *Tetrahymena, Euglena, Dictyostelium,* and bacteria as well as erythrocytes tolerate extreme gas supersaturations without bubbles forming internally (Hemmingsen and Hemmingsen, 1978, 1979, 1983; Hemmingsen *et al.,* 1985). Generally the cells are unaffected by nitrogen supersaturations up to 150 atm—and many even by supersaturations in excess of 200 atm—which is sufficient to cause profuse spontaneous formation of bubbles in water and aqueous solutions (Hemmingsen, 1977). Although *Euglena, Dictyostelium,* and *Tetrahymena* eventually show signs of damage (loss of colony-forming ability or decrease in number of cells), bubbles have been observed to form only in cells of *Tetrahymena.* These intracellular bubbles occur when the cells contain food vacuoles, which may act as nucleation sites, but only at gas supersaturations approaching the threshold for spontaneous nucleation in water (Hemmingsen, 1982; Hemmingsen and Hemmingsen, 1983).

Because the cell types that have been examined so far are relatively small (7000 μm^3 or less) and since the cell interior constitutes a somewhat isolated environment, it has been speculated (Hemmingsen *et al.,* 1985) that the underlying cause of the remarkable tolerance of cells to gas supersaturation is a lack of sufficient quantities of intracellular water. This view assumes that the spontaneous formation of a nucleus must be supported by a surrounding body of water of minimum size for a given content of dissolved gas.

This investigation was undertaken to obtain some evidence that the availability of water and cell size may be important factors affecting the nucleation of bubbles in cells. Eggs and early embryonic stages of the sea urchin *Lytechinus pictus* were selected for these experiments. The eggs are easily collected, fertilized and raised, and all stages up to metamorphosis are relatively transparent. Also, the eggs have a much larger volume (approximately 500,000 μm^3) than the cells used previously.

Other investigations into the effects of gas supersaturating conditions on fish and crustacean larvae have indicated that the younger organisms are more resistant to bubble formation (McDonough and Hemmingsen, 1984, 1985; Gray *et al.,* 1985). Our goal was to test sea

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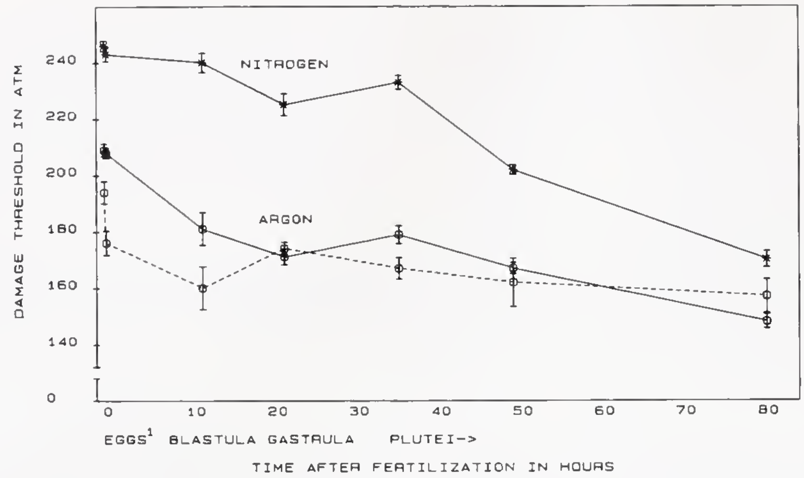

Figure 1. Mean gas supersaturation thresholds for damage to sea urchin eggs and early development stages. The means were computed using the midvalue of a range of pressures that bracketed the significant damage threshold. When the pressures tested did not provide values above and below the 40% significance level the pressure with damage closest to the significance level was used in computing the mean. Two methods of agitation were used during these experiments; shaken samples are represented by a solid line and stirred samples with a stippled line. Error bars represent the standard error. ¹The upper left values at time zero of each line represent unfertilized eggs while the values to the lower right, just off the zero of each line, represent eggs tested approximately 5 minutes after fertilization.

urchins' tolerance to gas supersaturation throughout the developmental process, and to attempt to correlate any changes in the damage threshold with morphological developments such as the subdivision of the cytoplasm or the formation of the blastocoel. Because early embryonic stages do not feed, the transition to a feeding larval stage enabled us to examine the impact of the ingestion of potential nucleating agents, such as gas nuclei or particles, on the supersaturation tolerances of the larvae.

Materials and Methods

The eggs of the sea urchin *Lytechinus pictus* were collected by intracoelomic injection of 0.5 *M* KCl, rinsed twice in seawater, and kept in a water bath at 16–18°C until used. Sperm were collected in a similar manner, concentrated, and stored on ice until used. Fertilized eggs and embryos were kept in a beaker of seawater at room temperature (21–25°C) (Hinegardner, 1975; Hinegardner and Rocha Tuzzi, 1981). Debris and dead embryos were removed daily from the beaker with a pipette; 10–20 ml fresh seawater was added. Unfertilized and fertilized eggs, blastula, gastrula, and pluteus stages of *L. pictus* were used in the experiments.

The threshold for damage and/or bubble formation was tested for each developmental stage by exposing 1 to 2 ml seawater containing the organisms to various gas saturations, followed by decompression. The experimental apparatus is described elsewhere (Hemmingsen and Hemmingsen, 1978, 1979). The small pressure chamber containing the sample was agitated by shaking (3 cycles/s) or by magnetic stir bar (*ca.* 200 rpm) during the gas exposure while a duplicate sample was simultaneously agitated in air at ambient pressure (1 atm). A 30-min equilibration time was used for most experiments; additional time for equilibration did not affect the threshold for damage, except for the unfertilized eggs subjected to shaking. In this latter case 1 to 2 hour equilibration times were used. After equilibration, the gas pressure in the chamber was rapidly released with complete decompression occurring within 2 s (fast decompression). In some experiments the gas was discharged slowly in steps at rates varying from 25 to 100 atm every 10 min (slow decompression). These slow decompression experiments were performed to reduce gas supersaturation, thereby separating its effects from others inherent in the procedure such as hydrostatic pressure, hypoxic conditions, and the gases themselves, all of which are potentially damaging.

At the beginning and end of each experiment on stages through the blastula, three subsamples of the stirred suspensions were collected in glass capillaries (75 mm × 1.1 or 1.5 mm) to count (at 75–150× in a compound microscope) the number of intact organisms per unit volume (Hinegardner, 1975) and to assess the degree of damage sustained. Six subsamples were used for the gastrula and later stages to compensate for the decreased number of

Table I

Comparison of bubble occurrence and frequency

Experimental conditions	Developmental stage	Total # experiments	Pressure range tested	# Experiments with internal bubbles[a]	Bubble occurrence pressure range	% Experiments with internal bubbles
Argon shaken	unfertilized eggs	60	175–220	19	210–220	32
	fertilized eggs	56	195–220	23	195–215	41
	blastulae	9	165–185	1	185	11
	gastrulae	17	165–195	4	165–185	24
	plutei	25	145–185	6	165–185	24
Nitrogen shaken	unfertilized eggs	13	230–250	1	240	8
	fertilized eggs	12	230–250	5	240–250	42
	blastulae	10	220–250	0	N/A	0
	gastrulae	17	200–240	1	230	6
	plutei	21	160–240	9	170–240	43
Argon stirred	unfertilized eggs	18	150–205	2	195–205	11
	fertilized eggs	8	155–185	0	N/A	0
	blastulae	8	145–175	1	165	13
	gastrulae	20	155–185	1	175	5
	plutei	46	145–185	13	155–185	28

[a] Defined as an experiment in which at least one organism was found to contain an internal bubble.

organisms per unit volume. Bubbles frequently formed in the capillary tubes filled with post-decompression subsamples. Repeated inversion of the capillary removed the bubbles and permitted a clear view of the contents and accurate determination of the volume. After the capillary subsamples had been removed, a few organisms were pipetted onto slides from the stirred suspensions and immediately observed. These observations were augmented by further examination of the capillary subsamples, which were first used for quantitative analysis then transferred to slides. This permitted examination of organisms and bubbles within 1 min of decompression and then 30–60 minutes later after the capillary contents had been counted.

An organism was considered damaged and therefore was not counted as having survived decompression if it exhibited any of the following characteristics: (1) obvious leakage of cytoplasm; (2) very dense or grainy appearance, especially in the early stages; (3) ragged or torn membranes or tissues, especially in the plutei; or (4) fragmentation. A "significant" level of damage was a 40% difference in the capillary counts following decompression [% difference = (# organisms reference − # organisms after)/# organisms reference × 100]. The reference value for the number of organisms in a sample used in this calculation represents a baseline value established from the arithmetic mean of a series of capillary counts made before the experiments from the stock culture and after decompression from the unpressurized control samples. The multiple samples taken from the stock culture during any series of experiments displayed no systematic variability. The level of significance is the point at which 40% or more of the total number of organisms were damaged according to the criteria given above. This 40% level also corresponded to the onset of severe damage.

One difficulty with the capillary counting method is that when small numbers of organisms were present, the method would occasionally (less than 10% of the time) yield a larger number of organisms after the experiment than before. When the percent difference numbers calculated from these values were required for additional calculations, they were set equal to zero because the negative values for the percent difference were obviously an artifact. Since this error appeared largely in experiments on small populations below threshold levels, it was rarely a factor in determining the damage threshold values.

Results

All developmental stages showed a remarkably high tolerance to gas supersaturation. The threshold for inducing damage in eggs is in excess of 240 atm nitrogen and 200 atm argon. Even larval plutei can tolerate more than 170 atm nitrogen and 140 atm argon ([illegible]). In most experiments performed above the threshold level more than one-third (range 10–100%) of the remaining organisms, apparently undamaged and often bubble-free, slowly disintegrated within 5 to 10 minutes following decompression. In these organisms which were intact after decompression, one or more bubbles were sometimes (in about 25% of the experiments) present. Bubbles

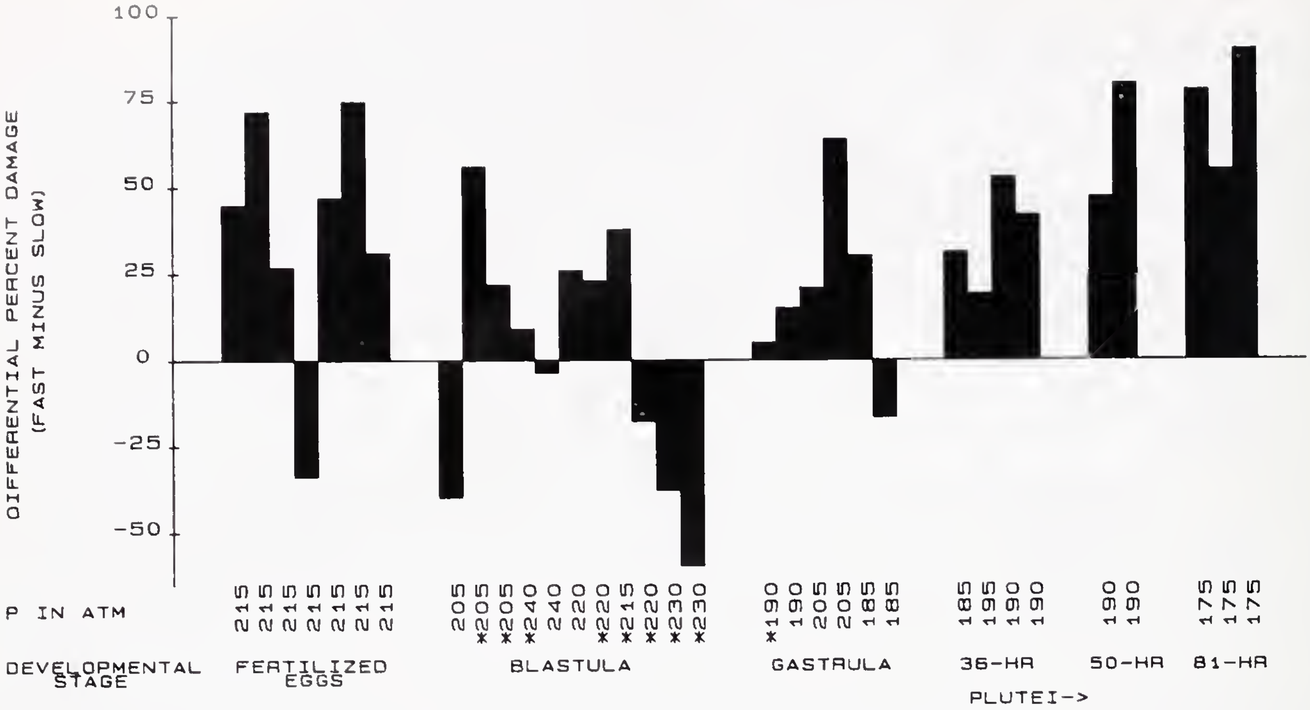

Figure 2. Comparison of damage inflicted on sea urchin eggs and early development stages by either fast or slow decompressions. The percent damage sustained during a fast decompression trial minus the percent damage of a slow decompression trial is plotted as the differential percent damage. A paired fast and slow decompression were performed on a sample from the same stock culture and equilibrated to the same final pressure. The decompression rate for the slow experiments was 50 atm every 10 minutes except for those bars marked with an asterisk (*) in which the slow decompression rate was 100 atm every 10 minutes.

were found in all developmental stages, but the frequency of their occurrence varied with the type of gas used, method of agitation, level of gas supersaturation, and stage of development (Table 1). The minimum gas supersaturation required for bubble formation was 155 atm argon for plutei, but went as high as 250 atm nitrogen for fertilized eggs. Multiple internal bubbles were observed exclusively in eggs and then only when nitrogen was used; no more than three bubbles per egg were ever observed.

The level of gas supersaturation required for damage generally decreased as the embryos developed (Fig. 1). With either argon or nitrogen the threshold decreased as the eggs developed into the blastula or gastrula stage, then increased slightly before consistently decreasing through the pluteus stages. The overall damage threshold was 20 to 50 atm higher when nitrogen gas was used in place of argon.

Slow decompressions generally were less damaging to the organisms than fast decompressions (Fig. 2). With the egg, gastrula, and pluteus stages the fast decompressions damaged large numbers of organisms in at least 80% of the experiments performed. However, for the blastula stage only 45% of the fast decompression experiments resulted in more damage than the slow decompressions.

The thresholds for damage of the unfertilized and fertilized eggs were significantly ($P = .95$, two tailed t-test) affected by the method of agitation used; frequently samples equilibrated by stirring yielded a lower threshold for damage than those equilibrated by shaking agitation (Fig. 1). Since this difference could be associated with equilibration times, the effect of prolonged equilibration with argon on eggs was examined (Fig. 3). Extending the shaking agitation of fertilized eggs from 1 to 2 hours had little effect whereas unfertilized eggs sustained more damage. Because this effect had little bearing on our main objectives it was not explored further.

Discussion

The sea urchin eggs and early developmental stages all displayed striking tolerances to gas supersaturation, similar to those of many eucaryotic microorganisms, and

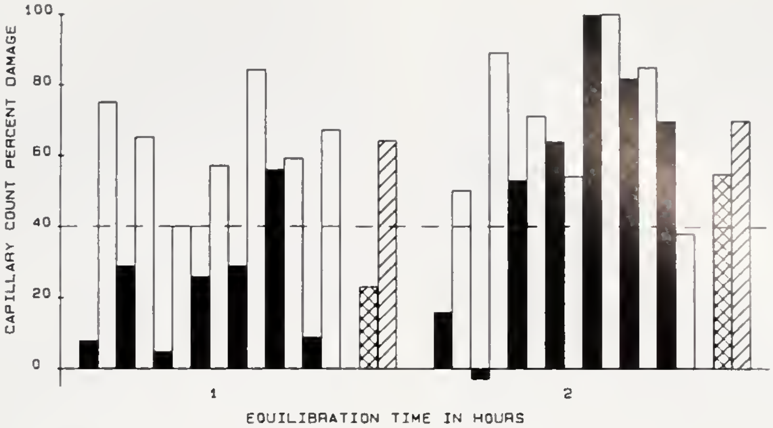

Figure 3. Damage to unfertilized and fertilized eggs following equilibration with argon at 210 atm for 1 and 2 hour with subsequent rapid decompression. Each bar represents the percent damage incurred by unfertilized eggs (solid bars) and fertilized eggs (open bars) in 14 separate trials. The mean values for damage sustained at each equilibration length are reported for unfertilized eggs (cross-hatched bars) and fertilized eggs (slashed bars).

much higher than those obtained for other invertebrates and vertebrates (Hemmingsen and Hemmingsen, 1979, 1983; McDonough and Hemmingsen, 1984, 1985). Also, in most cases the spread between supersaturations which produce slight damage and high survival and those which produce consistant damage and low survival is quite narrow (less than 10 atm). The resistance of the egg cells to bubble formation indicates (1) a lack of pools of free water of sufficient size to support spontaneous bubble nucleation in spite of the considerable size of the eggs (about 100 μm in diameter); (2) that a limited overall cell volume is not a prerequisite for obtaining the extraordinary resistance to bubbles that has been observed in most of the unicellular organisms studied so far; and (3) that the intracellular environment is void of hydrophobic interfaces in direct contact with the aqueous phase, since such interfaces are likely to destabilize the intracellular environment thereby creating conditions that favor nucleation.

In general there is a decrease in the threshold for damage during embryonic development. Internal bubbles developed in the eggs and in the other stages at supersaturations equal to or higher than the thresholds for damage (compare Fig. 1 and Table I); for the eggs and the blastulae, these gas supersaturations were substantially higher than those required for spontaneous nucleation of bubbles in water (Hemmingsen, 1977). The formation of intracellular bubbles in the eggs is an unusual phenomenon. Among the numerous types of cells studied, bubbles have previously been observed only in the ciliate *Tetrahymena* containing food vacuoles. The formation of bubbles in *Lytechinus* eggs requires gas supersaturations greater than those for spontaneous nucleation of bubbles in water (Hemmingsen, 1977); those in *Tetrahymena* form with gas supersaturation levels somewhat below the threshold for water, possibly because of particle surfaces within the food vacuole (Hemmingsen and Hemmingsen, 1983).

The presence of the fluid-filled blastocoel was expected to cause the blastulae to have a damage threshold more similar to that of bulk water, since the cavity contains a relatively homogenous liquid lacking obvious macroscopic structure (Stearns, 1974). This fluid could then act as a pool of "free" water with normal nucleation properties, however this characteristic was not readily apparent. The blastulae actually exhibited the lowest mean frequency of internal bubbles and had a higher threshold for damage than most of the subsequent developmental stages (Fig. 1, Table I). The large standard error and variability (Figs. 1, 2) of the values associated with this stage made finer distinctions difficult.

The gastrula is a stage in rapid transition that appears to be slightly more susceptible to damage by gas supersaturation than the stages preceding or immediately following. It is unlikely that the damage threshold of this stage is dependent on the presence of skeletal rudiments (spicules) as they represent an incomplete framework and are rarely seen protruding from the organisms following decompression. The formation of the alimentary canal at this stage involves the inward migration of cells and the movement and attachment of cells via pseudopodia (Giudice, 1973). Although the introduction of these structures divides the internal fluid into smaller entities which may be more stable, it is also possible that the restructuring of this internal environment introduces new, destabilizing interfaces that may enhance nucleation.

The transition to a feeding larva is one of the major changes that occurs during the pluteus stage. When the plutei begin feeding, ingestion of gas micronuclei and particulate substances from the seawater could promote bubbles in the digestive tract which upon expansion would damage the organisms. Indeed, many of the bubbles observed in the plutei were in the region of the mouth, and some otherwise intact plutei had a damaged alimentary canal. The presence of a complete internal skeleton may also be important in determining the damage threshold for this stage. Slight protrusion of the skeleton is minimally important to the long-term survival of the plutei following decompression because repair occurred within 24 hours. Extrusion of the skeleton became more critical as we approached the damage threshold for the plutei and the numbers of internal bubbles increased. This increased the stretching of the tissue and the likelihood that the skeleton would puncture and rip it at stress points. It is thus in relation to the rigid framework provided by the skeleton and the stretching imposed on the tissues by internal bubbles that the presence of a skeletal framework may affect the threshold for damage.

Overall, the fast and slow decompression experiments conducted on the plutei were more consistent than for other stages. This might partially reflect the fact that the initially heterogeneous group of embryos were refined by the stressful conditions of the culture so that only the strongest and healthiest survived to the pluteus stage. Also, this stage has a skeleton and other internal structures that may help to stabilize it against the impact of both external and internal bubbles.

The damage observed in all stages was largely caused by the gas supersaturation produced by rapid decompression, since the slow decompressions, which eliminated most of the gas supersaturation and related bubble formation, generally resulted in less damage (Fig. 2). The exception is the blastula stage (Fig. 2) for which the prolonged exposure necessary for the slow decompressions appeared to have a detrimental effect. The slow decompression data for all other stages show that they are not much affected by the gas exposure *per se,* the hydrostatic pressure, or the hypoxic conditions inherent in the experimental procedure.

Whereas fast decompression from threshold levels or greater yielded debris and large pieces of tissue from organisms that had ruptured during decompression, slow decompressions from the same levels resulted only in organisms that were grainy and wrinkled but otherwise undamaged; little debris was evident. This suggests that the damage suffered during the slow decompression is largely due to factors intrinsic to the procedure, such as the length of gas exposure, rather than to bubble formation. The slow decompression experiments were not conducted on the unfertilized eggs because the duration and number of experiments required would have reduced the integrity of these eggs, as they usually begin to disintegrate a few hours after spawning even at atmospheric pressure.

The significant threshold differences observed for the early stages when comparing the two methods of agitation (Fig. 1, Table I) may be the result of a higher sensitivity of these stages to the shear forces present when stirring. For the egg stages though, these threshold differences extend to shaking experiments when shorter equilibration times were compared with those longer than 1 hour. The cause of these time-dependent threshold differences has not been determined. Some evidence suggests that there are different permeabilities for the membranes of unfertilized and fertilized *Lytechinus* eggs with respect to gases since permeability differences have been reported for other substances such as water and ethylene glycol (Lillie, 1916; Stewart and Jacobs, 1932). However, recent investigations (Merta *et al.*, 1986) suggest that differences such as these may actually depend on the character and quantity of the intracellular water present in unfertilized *versus* fertilized eggs. Further studies are required to resolve this question of equilibration differences.

Acknowledgments

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A. Journal abbreviations, and book titles, all underlined (for *italics*)

B. All components of abbreviations with initial capitals (not as European usage in WORLD LIST *e.g. J. Cell. Comp. Physiol.* NOT *J. cell. comp. Physiol.*)

C. *All* abbreviated components must be followed by a period, whole word components *must not* (*i.e. J. Cancer Res.*)

D. Space between all components (*e.g. J. Cell. Comp. Physiol.*, not *J.Cell.Comp.Physiol.*)

E. Unusual words in journal titles should be spelled out in full, rather than employing new abbreviations invented by the author. For example, use *Rit Vísindafjélags Islendinga* without abbreviation.

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Reference: *Biol. Bull.* **174**: 91–94. (April, 1988)

Marine Biological Laboratory Centennial Calendar*

APRIL

29 **A Panel Discussion on Learning and Memory.** Speakers include Daniel Alkon, Jerome Kagan, and David Hubel. 7:30 pm, Lillie Auditorium.

MAY

22–25 **The Cellular Basis of Morphogenesis symposium** honoring Dr. J. P. Trinkaus on his retirement from Yale University. See schedule below.

24 **Futures in Science Student Presentations.** Noon, Candle House 104/105.

JUNE

9, 10 **MBL Update.** Science writers and Science Writing Fellowship alumni meet to learn about and discuss science as it is performed at the MBL.

23–25 **Ionic Channels: Structure, Function, and Morphology symposium.**

24 **Centennial Evening Lecture: Meredithe Applebury,** speaker. 8:00 pm, Lillie Auditorium.

26 **Scientific Illustration—1560–1988 exhibit.** Lillie Conference Room. 6/26–7/10.

30 **Cape Cod: A Diversity of Life** photo contest and exhibit. Meigs Room, Swope Center. 6/30–7/15.

JULY

1 **Centennial Evening Lecture: Daniel Koshland,** speaker. 8:00 pm, Lillie Auditorium.

1, 2 **"To See What Everyone Has Seen, To Think What No One Has Thought."** A symposium honoring the late Albert Szent-Györgyi. See schedule below.

8 **Centennial Evening Lecture (Lang Lecture): Torsten Wiesel,** speaker. 8:00 pm, Lillie Auditorium.

14, 15 **Centennial Evening Lecture: Joshua Lederberg,** speaker. 8:00 pm, Lillie Auditorium.

15 **Albert M. and Ellen R. Grass Reference Room Dedication.** MBL/WHOI Library.

17 **Dedication Day** (the anniversary of the MBL's opening)

4:00 pm—Opening of the Centennial Art Exhibit, Meigs Room.

8:00 pm—Centennial Lecture: Gerald Weissmann, speaker. Lillie Auditorium.

21, 22 **Forbes Lectures: Sydney Brenner,** speaker. 8:00 pm, Lillie Auditorium.

29 **Centennial Evening Lecture: Clay Armstrong,** speaker. 8:00 pm, Lillie Auditorium.

AUGUST

4 **Cape and Islands Chamber Music Festival Concert.** 8:00 pm, Lillie Auditorium.

5 **Centennial Evening Lecture: John Hobbie,** speaker. 8:00 pm, Lillie Auditorium.

6 **National Academy of Sciences reception**

Monsanto Biotechnology Lecture. Donald Kennedy, speaker. 8:00 pm, Lillie Auditorium.

7 **Hiroshima Day Lecture: Robert Solow,** speaker. 8:00 pm, Lillie Auditorium.

11 **Reflections with Katsuma Dan and Daniel Mazia**

12–19 **MBL Centennial Celebratory Week:**

12 **Corporation Meeting**

Trustees' Meeting

Opening Ceremony

Centennial Evening Lecture: E. O. Wilson, speaker

13 **Computational Neuroscience media event**

Dedication of Charles Ulrick Bay Reading Room

14 **Open rehearsal for Gala Concert**

Gala MBL Centennial Concert featuring new composition by Ezra Laderman. Performed by Jean-Pierre Rampal, Jelle Atema, and the Colorado String Quartet.

15 **Biomedical Applications of Basic Research symposium**

Community Day

16 **Centennial Evening Lecture: Clifford Slayman,** speaker

17 **Old Timers' Day**

19 **Centennial Evening Lecture: Shinya Inoué,** speaker

Closing Ceremony

20 **Cape and Islands Chamber Music Festival Concert.** 8:00 pm, Lillie Auditorium.

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* Schedule is subject to change.

22–24 MBL General Scientific Meetings.
26 Centennial Evening Lecture: John Gurdon, speaker. 8:00 pm, Lillie Auditorium.
27–30 Developmental Biology of Sea Urchins symposium.

SEPTEMBER

3–6 Ionic Controls in Development symposium. See schedule below.

THE CELLULAR BASIS OF MORPHOGENESIS

22–25 May 1988
Marine Biological Laboratory,
Woods Hole, Massachusetts

Sunday, May 22	Dinner followed by a wine and cheese party
Monday, May 23	CELL MOTILITY IN CULTURE
Morning	Cell movement *in vitro*
	C. Izzard (SUNY Albany)
	J. Couchman (U. Alabama)
	B. Geiger (Weizmann Inst.)
	W.-T. Chen (Georgetown U.)
	V. Small (I. fur Molekularbiologie)
Afternoon	Biochemistry of the cytoskeleton
	K. Berridge (UNC, Chapel Hill)
	J. Spudich (Stanford U.)
	T. Stossel (Mass. General Hosp.)
	L. Taylor (Carnegie Mellon U.)
	G. Oster (UC Berkeley)
Evening	Poster Session
Tuesday, May 24	CELL MOTILITY IN THE ORGANISM
Morning	Control of Directional Movement
	S. Zigmond (U. Pennsylvania)
	D. McClay (Duke U.)
	T. Poole (SUNY, Syracuse)
	K. Johnson (George Washington Med. Sch.)
	R. Keller (UC Berkeley)
Afternoon	Patterning by the embryonic environment
	K. Tosney (U. Michigan)
	D. Bentley (UC Berkeley)
	C. Wylie (St. George's Med. School)
	C. Erickson (UC Davis)
	J. P. Trinkaus (Yale U.)
Evening	Banquet in honor of J. P. Trinkaus
Wednesday, May 25	Breakfast and departure

For further information contact: Carol Erickson, Department of Zoology, University of California, Davis, CA 95616.

"TO SEE WHAT EVERYONE HAS SEEN, TO THINK WHAT NO ONE HAS THOUGHT."

—Albert Szent-Györgyi

A symposium in his memory and honor

1, 2 July 1988

Marine Biological Laboratory,
Woods Hole, Massachusetts

Friday, July 1	
8:30–9:00	**Benjamin Kaminer** (on Szent-Györgyi)
9:10–9:50	**Michael Brown**
10:05–10:30	Break–Refreshments
10:30–11:10	**Joseph Goldstein**
11:25–12:05	**Michael Berridge**
12:20–1:25	Lunch
1:30–2:10	**Gottfried Schatz**
2:25–3:05	**Aaron Klug**
3:25–3:50	Break–Refreshments
3:50–4:30	**Hugh Huxley**
5:00–6:00	Refreshments
8:00–9:00	**Daniel Koshland**
9:00	Wine & Cheese
Saturday, July 2	
9:00–9:40	**David Baltimore**
9:55–10:35	**Phillip Leder**
10:55–11:20	Break Refreshments
11:20–12:00	**Carlton Gajducek**
12:30	Lunch
6:00	Refreshments and Banquet

Chairpersons and official discussants include Laszlo Lorand, Hans Kornberg, Andrew Szent-Györgyi, Setsuro Ebashi, A. M. Weber, George Wald, Michael Kasha, Harlyn Halvorson, Salvador Luria, John Gergely, Ronald Pethig, and Denis Robinson.

For further information contact: Benjamin Kaminer, Department of Physiology, Boston University School of Medicine, 80 E. Concord Street, Boston, MA 02118-2394.

DEVELOPMENTAL BIOLOGY OF SEA URCHINS V

27–30 August 1988

Marine Biological Laboratory
Woods Hole, Massachusetts

Saturday, August 27	
3:00 pm	Registration—Swope Center
5:00 pm	Mixer—Swope
7:30 pm	SESSION I. CELL INTERACTIONS AND MORPHOGENESIS

William Lennarz (M.D. Anderson, University of Texas), Chairman

Charles Glabe (Univ. of California, Irvine)
Fred Wilt/Steve Benson (Univ. of California, Berkeley)
Dave McClay (Duke University)
Charles Ettensohn (Carnegie-Mellon Univ.)
Robert Simpson (NIH)

Sunday, August 28
8:30 am SESSION II. MOLECULAR ASPECTS OF MOTILITY

Bruce Brandhorst (McGill University), Chairman

David Burgess (University of Miami)
Richard Vallee (Worcester Foundation)
David Asai (Purdue University)
Ray Stephens (MBL, Woods Hole)
Martin Nemer (Institute for Cancer Research, Fox Chase)
Victor Vacquier (Univ. of California, San Diego)

3:00–6:00 pm POSTER SESSION
5:00–6:00 pm Cocktails
7:30 pm SESSION III. EGG ACTIVATION

Bennett Shapiro (Univ. of Washington), Chairman

David McCullough (Univ. of Miami)
Laurinda Jaffe/Fraser Shilling (Univ. Connecticut/Univ. S. Cal)
William Kinsey (Univ. of Miami)
Alina Lopo (Univ. California, Riverside)
Rob Swezey (Hopkins Marine Stn., Stanford)

Monday, August 29
8:30 am SESSION IV. LINEAGES AND GENE EXPRESSION

Bob Angerer (Univ. of Rochester), Chairman

Henry Sucov (Cal. Tech)
Rudy Raff (Indiana University)
Gary Wessel (Univ. of Texas, M.D. Anderson)
Andy Cameron (Cal. Tech)
Bob Burke (Univ. of Victoria)
Bill Klein (Univ. of Texas, M.D. Anderson)
Greg Wray (Indiana University)

3:00–6:00 pm POSTER SESSION
5:00–6:00 pm Cocktails
7:30 pm SESSION V. REGULATION OF GENE EXPRESSION

Rob Maxson (Univ. S. California), Chairman

Geoffrey Childs (Albert Einstein, New York)
Eric Weinberg (Univ. of Pennsylvania)
Bill Marzluff (Fla. State University)
Frank Calzone (Cal. Tech)
Matt Winkler (Univ. of Texas)

Tuesday, August 30
8:30 am SESSION VI. ACTIVATION OF THE NUCLEUS AND MITOSIS

Gerald Schatten (Univ. of Wisconsin), Chairman
Hikoichi Sakai (University of Tokyo)
Roger Sloboda (Dartmouth)
Ryoko Kuriyama (Univ. of Minnesota)
Edward Salmon (Univ. North Carolina, Chapel Hill)
Hidemi Sato (Nagoya University)
Christian Petzelt (German Cancer Research Center)
John Scholey (National Jewish Hospital, Denver)

For further information contact: David R. McClay, Department of Zoology, Duke University, Durham, NC 27706.

IONIC CONTROLS OF DEVELOPMENT

Very Tentative Program
(Additions and Title Changes Expected)

3–6 September 1988

Marine Biological Laboratory,
Woods Hole, Massachusetts

Sunday, September 3
3–5 pm Registration
5 pm Social Hour
6 pm Dinner

7:30–10 pm SESSION 1: CALCIUM CONCENTRATION GRADIENTS IN DEVELOPMENT

Wiel Kuhtreiber (Marine Biological Laboratory)
"A vibrating Ca^{2+} electrode for detecting extracellular Ca^{2+} gradients"

Darryl Kropf (Oregon State University)
"Are Ca^{2+} gradients required for *Fucus* polarization?"

Lionel Jaffe (Marine Biological Laboratory)
"Calcium gradients are required for polarization in *Fucus* eggs"

Martin Steer (University College Dublin)
"Calcium control of pollen tube tip growth"

Shun-ichi Miyazaki (Jichi Medical School)
"Calcium waves in activating hamster eggs"

Monday, September 4
8:30 am–12:30 pm SESSION 2: ION CURRENTS AND CELL POLARITY IN PLANT SYSTEMS

William Lucas (University of California, Davis)
"Cellular mechanisms controlling the extracellular current pattern in *Chara* internodal cells"

Neil Gow (University of Aberdeen)
"The circulating currents in water moulds may relate to localised nutrient uptake, not cell polarity"

Cynthia Troxell (University of Colorado)
"Ionic currents present during tip growth in desmids"

Mary Jane Saunders (University of South Florida)
"Ionic currents during division of filamentous plant cells"

Robyn Overall (University of Sydney)
"Induction of new polarities in lettuce pith"

Keerti Rathore (Purdue University)
"Ionic basis of currents in developing somatic embryos of *Daucus carota*"

Thomas Bjorkman (University of Washington)
"Role of ionic currents in transduction of the gravity stimulus in plants"

Julia Hush (University of Sydney)
"Steady ionic currents around *Pisum sativum* roots after wounding"

Andrew Miller (University of Aberdeen)
"Ion currents and growth regulators in plant root development"

Mark Schiavone and Richard Racusen (University of Maryland)
"Ion asymmetries in carrot embryogenesis"

12–3 pm Lunch and free time

3–6 pm SESSION 3: ION CURRENTS AND CELL POLARITY IN ANIMAL SYSTEMS

Benjamin Peng (University of North Carolina)
"Ionic control of synaptic differentiation"

J. G. Kunkel (University of Amherst)
"Ionic currents during very early development of the cockroach oocyte"

Erwin Huebner (University of Manitoba)
"A 2-d vibrating probe analysis of the ionic currents in the ovary of *Rhodnius prolixus*"

Rene Dohmen (University of Utrecht)
"Ionic currents in early development of molluscs"

Glen Winkel (University of California, Davis)
"A 2-d vibrating probe analysis of the ionic currents around the gastrulating mouse embryo"

Steve Baumann (Environmental Protection Agency)
"Toxicological studies using the vibrating probe"

6:30 pm BANQUET

Tuesday,
September 5
9:00–12 pm SESSION 4: GALVANOTROPISM AND GALVANOTAXIS IN DEVELOPMENT

Hans Gruler (University of Ulm)
"Directed growth and movement of cells in asymmetric cellular environments"

Richard Nuccitelli (University of California, Davis)
"Mechanisms of signal transduction in neural crest cell galvanotaxis"

William Parkinson (University of Michigan)
"Experiments on the interaction of electromagnetic fields with mammalian systems"

Watt Webb (Cornell University)
"Cell surface receptor crowding"

Rosemary White (University of Sydney)
"Effects of steady electric fields on regenerating *Mougeotia* protoplasts"

Keerti Rathore (Purdue University)
"Applied electrical fields stimulate plant growth"

Neil Gow (University of Aberdeen)
"Applied electrical fields polarize the growth of mycelial fungi"

Mark Cooper (Yale University)
"Electrophoresis of charged intracellular molecules through gap junctions by externally applied electric fields"

12–3 pm Lunch and free time

3–6 pm Tours of National Vibrating Probe Facility

6:30 Dinner

8–10 pm Workshops discussing common technique problems and how to solve them

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Wednesday,
September 6
9 am–12 pm SESSION 5: WOUND HEALING AND REGENERATION CURRENTS

Kenneth Robinson (Purdue University)
"Ca^{2+} gradients in regenerating neurons"

Richard Borgens (Purdue University)
"Mammalian nerve cord regeneration is enhanced by imposed electric fields"

Michele Miller Bever (Purdue University)
"Axonal responses to applied electrical fields *in situ* in the glass catfish"

Gene McGinnis (Purdue University)
"Enhancement of peripheral nerve regeneration by imposed electrical fields"

Joseph Vanable (Purdue University)
"Effects of electrical fields on wound healing in amphibian skin"

12 noon Lunch and departure

For further information contact: Richard Nuccitelli, Department of Zoology, University of California, Davis, CA 95616.

Reference: *Biol. Bull.* **174:** 95–108. (April, 1988)

Intracellular Signal Transduction and Amplification Mechanisms in the Regulation of Oocyte Maturation

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The Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Abstract. Meiotic cell division can be induced by various extracellular signals in different organisms. The inducing signals interact with surface receptors. The signal is transduced across the oocyte plasma membrane, into the cytoplasm, where it is amplified by protein phosphorylation. Maturation-promoting factor appears in all meiotic and mitotic cells and is associated with protein phosphorylation. Cells use at least two systems for the activation of protein phosphorylation in response to extracellular stimuli: direct activation of a receptor-associated protein kinase and indirect activation via guanine nucleotide-binding proteins (G-proteins). G-proteins indirectly activate or inhibit protein kinases dependent on cAMP, Ca^{2+}, or diacylglycerol. Oocytes appear to use each of these signals and several different kinases to regulate meiotic cell division. The evidence for the involvement of each of these cellular signal transduction and amplification mechanisms in the regulation of meiotic cell division is discussed. Models are presented to account for possible interactions between various positive and negative modulators.

Introduction

Meiotic cell division is of fundamental biological importance. Meiosis is the process leading to the maturation of the egg and the generation of the haploid chromosome content necessary for the continuation of the species after fertilization. It can also be a useful model for processes involved in the regulation of mitotic cell division. Large populations of oocytes arrested at meiotic prophase can be obtained easily, are amenable to metabolic labeling and biochemical analysis, and can be stimulated to undergo germinal vesicle breakdown (GVBD) synchronously without artificially synchronizing the population. Furthermore, meiotic cell cytoplasmic extracts cause breakdown of somatic cell nuclei. Thus, at least some of the intracellular regulatory processes leading to GVBD can also initiate mitotic cell divisions.

The reinitiation of meiotic maturation (removal of the prophase arrest) is normally triggered by an external effector stimulus. Often, but not necessarily, this stimulus is hormonal. The hormonal signals initiating GVBD in amphibian and starfish oocytes have been clearly identified as progesterone and 1-methyladenine (1-MA), respectively (for reviews see Kanatani, 1973; Masui and Clarke, 1979; Meijer and Guerrier, 1985). Amphibian oocytes can also be induced to undergo GVBD by exposure to insulin, which evidently acts by binding to an insulin-like growth factor receptor (IGFR) (Maller and Koonz, 1981). The hormones initiate GVBD by interacting with a component of the oocyte surface and are ineffective if introduced directly into the cytoplasm. Thus the hormonal signal must be transduced across the plasma membrane into the cytoplasm, where it elicits the biological response. In the cytoplasm, the signal can be positively or negatively modulated.

In other species, fertilization triggers GVBD, as well as egg activation and subsequent development. Sperm, of course, fertilize the egg by interacting with its surface. Thus it is reasonable to postulate that the transduction, relay, and amplification mechanisms involved in eliciting GVBD may be similar in oocytes induced by fertilization and hormonal stimulation. Furthermore, in the species in which the normal extracellular signal for GVBD is unknown, the oocyte may also use similar intracellular transduction and amplification mechanisms.

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Invariably, there is a delay between the exposure of the oocyte to the stimulus for GVBD and its response. This delay may be several minutes or many hours long, depending on the species and the stimulus. A portion of this time period may be required for the generation of a sufficient number of hormone-receptor interactions (Bellé *et al.*, 1976; Masui and Clarke, 1979; Nemoto, 1982), but it is also followed by a period during which the hormone is no longer necessary, but prior to the onset of GVBD. In organisms such as *Urechis* and *Spisula*, in which fertilization provides the stimulus for GVBD, the time corresponding to the hormone-dependent period of starfish and frog oocytes is negligible since fertilization is a very rapid process. Yet there is a period of 3–4 min after fertilization during which the process of GVBD can be easily blocked, and this period is followed by another similar time period during which the process is essentially irreversible, but before GVBD (Tyler and Schultz, 1932; Allen, 1953; Carroll and Eckberg, 1986; Eckberg *et al.*, 1987). These delays suggest that time is required for the activation of intracellular signals and relays.

Recent research has begun to elucidate the mechanisms involved in transduction, relay, and amplification of signals in somatic cells. Most signals which elicit biological responses interact with receptors which are linked ultimately with molecules which regulate protein kinases or which possess protein kinase activity themselves. The former group includes protein serine/threonine kinases regulated by cyclic nucleotides, calmodulin (CaM), or diacylglycerol (DG) (see review by Edelman *et al.*, 1987). By contrast, members of the family of growth factor receptors have protein tyrosine kinase activity (see reviews by Hunter and Cooper, 1986; Hunter, 1987). The number of identified intracellular and membrane-bound protein kinases is increasing rapidly, and it has been proposed that they act as amplifiers and/or switches in many cellular responses (Hunter, 1987). Such coordinate and antagonistic action can provide a great deal of flexibility and sensitivity to the regulation of cellular responses. This article will discuss somatic cell transduction and amplification mechanisms and their relevance to mechanisms involved in eliciting GVBD. Specifically, it will discuss evidence for G-protein mediated signal transduction and the involvement of specific protein kinases in the initiation of oocyte maturation.

Maturation-promoting Factor and Protein Phosphorylation

Cytoplasmic events occurring during the delays result in the appearance of maturation-promoting factor (MPF), an operationally defined activity that is always found in oocytes prior to GVBD (see Masui and Clarke, 1979, for review). Its presence is defined by the ability of the cytoplasm in which it is contained to cause recipient oocytes to mature rapidly and amplify the MPF (Masui and Markert, 1971). MPF is not tissue- or species-specific since an identical activity appears in all cells during the G_2 and M phases of the cell cycle (Kishimoto and Kanatani, 1976; Sunkara *et al.*, 1979; Kishimoto *et al.*, 1984; Dorée *et al.*, 1983; Miyake-Lye *et al.*, 1983; Gerhart *et al.*, 1984; Picard *et al.*, 1985b, 1987). Because this activity is present at mitosis, it is also called M-phase-promoting factor.

The widespread occurrence of MPF strongly indicates that it must play a fundamental role in the regulation of cell division. It may act generally to induce chromosome condensation and/or nuclear envelope breakdown (NEBD), since it can also cause NEBD, spindle formation, and chromosome condensation in somatic nuclei exposed to it *in situ* (Miyake-Lye *et al.*, 1983) and *in vitro* (Lohka and Maller, 1985). Although MPF can elicit these responses, it apparently does not do so directly because it can be separated from the factors directly responsible for these responses based on its failure to bind to nuclei *in vitro* (Newport and Spann, 1987).

MPF activity is invariably associated with protein phosphorylation. Microinjection of partially purified preparations of MPF causes an immediate increase in protein phosphorylation (Wu and Gerhart, 1980). Stage-specific protein phosphorylations occur in amphibian eggs and in cell-free extracts of amphibian eggs in response to MPF (Maller *et al.*, 1977; Karsenti *et al.*, 1987; Lohka *et al.*, 1987a).

MPF is stabilized by phosphatase inhibitors and labile in the presence of Ca^{2+} (Wu and Gerhart, 1980). Stabilization by phosphatase inhibitors indicates that the active form of MPF may be phosphorylated. The Ca^{2+} sensitivity could result from activation of a Ca^{2+}-dependent protease or phosphatase. It could provide a mechanism for the disappearance of MPF after cell division.

Initial studies using gel filtration revealed an apparent molecular weight of roughly 100 kDa for MPF (Wu and Gerhart, 1980). Recently, it has been purified approximately 2000-fold from *Xenopus* eggs (Lohka *et al.*, 1987b). The fraction has protein kinase activity with broad substrate specificity and can cause nuclear breakdown and chromosome condensation *in vitro*. The fraction contains only two major polypeptides (45 kDa and 32 kDa) when assayed by SDS-PAGE. The 45 kDa polypeptide becomes phosphorylated in the presence of ATP. Whether the molecule with MPF activity that was partially purified by Wu and Gerhart is the same as that obtained by Lohka *et al.* has not been determined. It is possible that more than one macromolecule exists with MPF activity. Nevertheless, the close association of MPF activity with protein phosphorylation and its sensitivity to

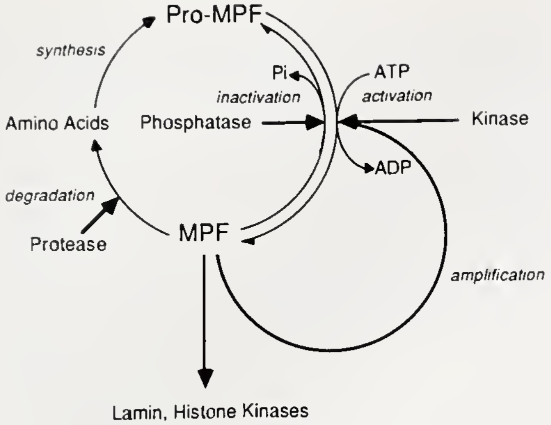

Figure 1. Scheme showing the proposed metabolism of MPF. MPF is synthesized as a precursor (Pro-MPF) which is activated by phosphorylation catalyzed by one or more protein kinases. It then amplifies its activity autocatalytically by autophosphorylation. It also activates other kinases (*e.g.*, lamin and histone kinases) responsible for nuclear protein phosphorylation. It could be inactivated by dephosphorylation or by proteolytic degradation. Heavy lines terminating in arrows represent an activation process. Thin lines terminating in arrows represent chemical conversions. Italicized words indicate what is happening to MPF at each stage in the scheme.

phosphatases strongly indicates that MPF should be a protein kinase.

A scheme for the metabolism of MPF and its involvement in GVBD is shown in Figure 1. In this model, MPF is synthesized as an inactive precursor, Pro-MPF. It becomes active upon phosphorylation. MPF has kinase activity and amplifies its activity by autophosphorylation. It then activates other kinases which directly phosphorylate nuclear proteins, leading to nuclear envelope breakdown and chromosome condensation. MPF could be inactivated by dephosphorylation or by proteolytic degradation.

The mechanisms involved in the initial appearance of MPF remain to be explained. Clearly, though MPF can be amplified autocatalytically, its initial activity must be turned on or activated by some process derived from earlier events in the GVBD triggering process. Since MPF amplification is invariably associated with protein phosphorylation and MPF is stabilized by phosphatase inhibitors, its initial activation probably involves phosphorylation as well. It is not known which if any specific phosphorylation sites on the molecule are essential for its activation, but both protein tyrosine and protein serine/threonine kinases can lead to its activation. The protein tyrosine kinase activity of IGFR appears to be essential to its activity in inducing GVBD (Morgan *et al.*, 1986). The possible induction of GVBD by protein tyrosine phosphorylation is further supported by the observation than microinjected pp60$^{v\text{-}src}$, an oncogenic protein tyrosine kinase, can accelerate GVBD in *Xenopus* oocytes (Spivak *et al.*, 1984). The other kinases that promote GVBD are protein serine/threonine kinases. A 42-kDa protein specifically phosphorylated at metaphase in *Xenopus* oocytes and oocyte extracts is phosphorylated on serine, threonine, and tyrosine residues (Lohka *et al.*, 1987a). This polypeptide has a molecular weight similar to that of a major phosphoprotein in highly purified MPF. The relationship between this molecule and MPF remains to be determined.

In principle, the disappearance of MPF activity could occur as the result of the action of either dephosphorylation or proteolysis. The stabilization of MPF by phosphatase inhibitors *in vitro* supports the idea that MPF is inactivated by dephosphorylation. However, the evidence suggests that dephosphorylation is not a general mechanism for the inactivation of starfish MPF *in vivo*. Co-microinjection of phosphatases into starfish oocytes does not block the amplification or activity of MPF (Meijer *et al.*, 1986). Furthermore, microinjection of the catalytic subunit of A-kinase, an antagonist of MPF production (see below), has no effect on the amplification or biological activity of MPF (Maller and Krebs, 1980). By contrast, cell cycle-specific proteolysis is known to occur (see below), and this activity may regulate MPF disappearance (Picard *et al.*, 1985b).

Protein phosphorylation "bursts" have been reported to precede GVBD in all species in which this phenomenon has been examined, including amphibians (Maller *et al.*, 1977), starfish (Guerrier *et al.*, 1977; Mazzei and Guerrier, 1982), molluscs (Eckberg *et al.*, 1987), echiuroids (Meijer *et al.*, 1982), and annelids (Peaucellier *et al.*, 1982, 1984). Some of the protein phosphorylation is undoubtedly due to the direct action of MPF, but other kinases are also implicated in specific protein phosphorylations. In some cases, these specific phosphorylations occur in response to MPF.

Ribosomal protein S6 is phosphorylated by two distinct kinases in *Xenopus* oocytes (Erikson and Maller, 1985, 1986; Erikson *et al.*, 1987) in response to hormonal stimulation or MPF microinjection (Hanocq-Quertier and Baltus, 1981; Nielsen *et al.*, 1982; Stith and Maller, 1984). There is no evidence that S6 phosphorylation has a causal relationship to GVBD. This ribosomal protein is phosphorylated in response to cell division and growth stimulating activities in a variety of cell types.

The phosphorylation of nuclear lamins and histones appears to be involved in GVBD and chromosome condensation. Lamin hyperphosphorylation occurs prior to NEBD (Gerace and Blobel, 1980; Ottaviano and Gerace, 1985; Newport and Spann, 1987) and provides an essential condition for NEBD. The lamin kinase is distinct from MPF. There is a lag between MPF addition and lamin hyperphosphorylation (Miake-Lye and Kirschner, 1985), and MPF, unlike the lamin kinase, does not bind to nuclei (Newport and Spann, 1987). Histone H1 phosphorylation also precedes GVBD (Hohmann *et al.*, 1976; Newport and Spann, 1987) and may provide an essential condition for chromosome condensation (Bradbury *et al.*, 1974; Inglis *et al.*, 1976).

G-proteins

Extracellular signals can regulate intracellular protein kinases indirectly by activating GTP-binding or G-proteins. A signal, the binding of a ligand to its cell surface receptor, causes the G-protein to exchange a bound GDP for GTP and become active by dissociation of the α from the β and γ subunits (Sternweis *et al.*, 1981). The activated α subunit may then regulate certain intracellular messenger systems which in turn regulate several specific cellular protein kinase activities indirectly. G-protein mediated cellular signal transduction has been recently reviewed (Stryer and Bourne, 1986). G-proteins are irreversibly activated by the binding of non-hydrolyzable analogs of GTP such as guanosine 5′-0-(3-thiotriphosphate) (GTP-γS) and 5′-guanylyl imidodiphosphate (GppNHp) and are blocked from being activated by the binding of guanosine 5′-0-(2-thiodiphosphate) (GDP-βS). The substituted guanine nucleotides can act on any of the G-proteins. By contrast, cholera toxin (CTX) and pertussis toxin (PTX) antagonize specific G-proteins. Therefore, these compounds are also used to probe for G-protein function.

Mechanisms of G-protein regulation of cell function which may be relevant for GVBD are summarized in Figure 2. This scheme offers several potential levels of positive and negative regulation of GVBD. Adenylate cyclase is regulated by at least two separate G-proteins, G_s and G_i. The GTP-bound α subunit of G_s (45 or 52 kDa) stimulates adenylate cyclase, whereas the GTP-bound α subunit of G_i (41 kDa) inhibits the enzyme (Northrup *et al.*, 1983; Katada *et al.*, 1986). Hydrolysis of GTP to GDP returns the G-proteins to their inactive state. CTX ADP-ribosylates G_s, decreasing its GTPase activity and thus maintaining it in its active state (Cassel and Selinger, 1978). Similarly, PTX blocks agonist-mediated inhibition of adenylate cyclase by ADP-ribosylating the α subunit of G_i (Katada and Ui, 1982). By regulating the activity of adenylate cyclase, these G-proteins control the cellular cAMP content and thus regulate indirectly the activity of cAMP-dependent protein kinase (A-kinase).

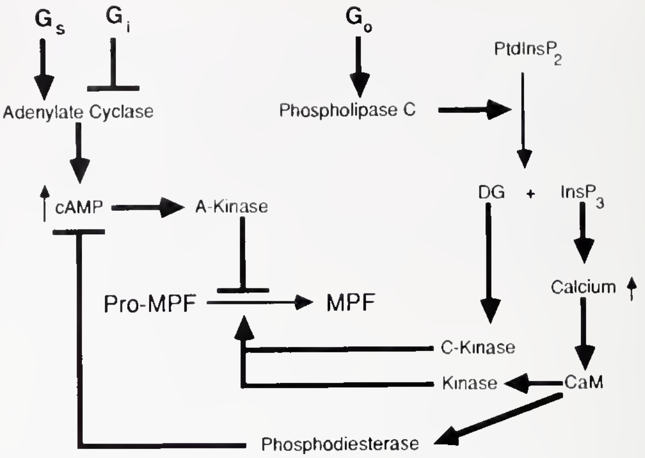

Figure 2. Possible regulatory mechanisms for G-proteins in GVBD. G_s and G_i regulate the content of cAMP, which regulates the activity of A-kinase, which antagonizes the conversion of Pro-MPF to MPF. G_o activates phospholipase C to hydrolyze phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$), producing diacylglycerol (DG) and inositol 1,4,5-trisphosphate ($InsP_3$). DG activates C-kinase which promotes the conversion of Pro-MPF to MPF. $InsP_3$ causes an increase in the cytoplasmic free calcium concentration due to release from intracellular stores. The elevated intracellular calcium activates calmodulin (CaM) which can activate a kinase which promotes the conversion of Pro-MPF to MPF, and/or it can activate phosphodiesterase which decreases the intracellular content of cAMP, thereby decreasing A-kinase activity. There is no direct evidence that MPF is a substrate for any specific kinase. However, it is not necessary for this model that the regulatory kinases act directly on Pro-MPF. There may be other intermediate substrates that act directly on Pro-MPF. In this and succeeding schemes, a heavy line ending in an arrow represents a stimulatory effect, a heavy line ending in a bar represents an inhibitory effect and a thin line ending in an arrow indicates a chemical conversion. Small vertical arrows adjacent to cAMP and Ca^{2+} indicate a cytoplasmic concentration increase.

Activation of another G-protein can activate a phosphatidylinositol-specific phospholipase C, resulting in the generation of inositol (poly)phosphates and diacylglycerol (Rodbell, 1980). The diacylglycerol activates a Ca^{2+}- and phospholipid-dependent protein kinase (C-kinase). The G-protein involved in this system could be G_o, the α subunit of which has an apparent molecular weight of 39 kDa. The distribution of G_o in mammalian brain parallels that of C-kinase (Worley *et al.*, 1986). Some of the inositol polyphosphates have Ca^{2+}-mobilizing activities, and the elevated Ca^{2+} concentration can activate other protein kinases, some of which are calmodulin (CaM)-dependent.

The evidence for the involvement of G-proteins in GVBD is strongest for frog and starfish oocytes. *Xenopus* oocytes have both the 45 and 52 kDa substrates of CTX and the 41 kDa substrate of PTX (Goodhardt *et al.*, 1984; Sadler *et al.*, 1984). In frog oocytes, CTX can block the induction of GVBD by progesterone (Godeau *et al.*, 1978). There is also some evidence that progesterone inhibits membrane-bound adenylate cyclase (Sadler and Maller, 1981) by blocking the action of a G-protein (Sadler and Maller, 1983). These data implicate the G_s in maintaining the oocyte in prophase meiotic arrest. They suggest that progesterone initiates GVBD by inactivating the G_s-protein, although the interpretation of some of

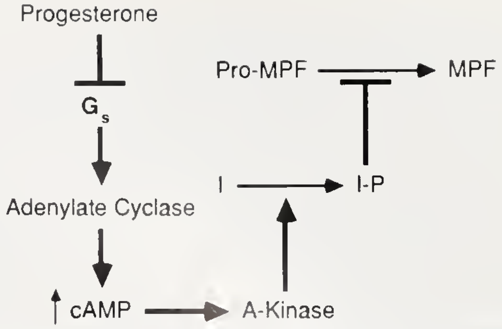

Figure 3. Possible scheme for induction of GVBD by progesterone in amphibian oocytes. Progesterone inactivates G_s which is coupled through adenylate cyclase and cAMP to A-kinase. A-kinase regulates GVBD by phosphorylating another molecule which inhibits the conversion of Pro-MPF to MPF. Thus the inhibitory action of A-kinase on the conversion of Pro-MPF to MPF and GVBD is blocked. Symbols are as in the legend to Figure 2.

their data in this way requires novel mechanisms of G-protein action (Sadler and Maller, 1985). If this interpretation is correct, CTX should irreversibly activate oocyte adenylate cyclase *in vitro*. It might also be possible to introduce PTX to block agonist-mediated reduction in the cAMP content due to activity of the G_i protein. However, CTX does not antagonize the inhibition of *Xenopus* oocyte adenylate cyclase by progesterone *in vitro*, and PTX has little effect on GVBD (Goodhardt *et al.*, 1984; Sadler *et al.*, 1984). Thus the exact involvement of G-proteins in progesterone-induced *Xenopus* oocyte GVBD is unclear.

Other G-proteins can also mediate GVBD in amphibians. Microinjection of transforming *ras* proteins, oncogene products with the characteristics of unregulated G-proteins, can elicit GVBD (Birchmeier *et al.*, 1985; Deshpande and Kung, 1987; Lacal *et al.*, 1987), and microinjection of a monoclonal anti-*ras* can block both progesterone-induced (Sadler *et al.*, 1986) and insulin-induced GVBD (Deshpande and Kung, 1987). Some studies suggest that *ras* proteins may elicit GVBD by interacting with the adenylate cyclase system. For example, CTX partially blocks the action of microinjected *ras* (Birchmeier *et al.*, 1985). However, *ras* protein microinjection has no effect on the intracellular cAMP content (Birchmeier *et al.*, 1985), and other studies show that *ras* protein microinjection activates another G-protein-mediated signal system (Lacal *et al.*, 1987). Therefore, the best evidence indicates that *ras* proteins elicit GVBD by a mechanism that is independent of action on adenylate cyclase.

In starfish oocytes, microinjected GDP-βS inhibits hormone-induced GVBD (Shilling and Jaffe, 1987). Additionally, microinjected (Shilling and Jaffe, 1987) or externally applied (Eckberg, unpub.) PTX inhibits hormone-induced GVBD. PTX inhibition of GVBD could be mediated through action on either G_i or G_o, assuming that starfish G-proteins have responses to these toxins which are similar to those of mammalian cells.

There is also some evidence that G-proteins regulate GVBD in *Spisula* oocytes. Serotonin is a G-protein agonist in other systems (Litosch *et al.*, 1985), and serotonin can elicit GVBD in *Spisula* oocytes (Sato *et al.*, 1985). There is direct evidence for activation of a phosphatidylinositol-specific phospholipase C after fertilization (Eckberg and Szuts, 1987; Bloom *et al.*, submitted). However, preliminary experiments with PTX were inconclusive. Externally applied PTX had no effect on *Spisula* oocytes, but we could not demonstrate that the antagonist entered the oocytes (Eckberg, unpub.).

In *Chaetopterus*, the evidence for G-protein signaling in the initiation of GVBD is more indirect. Microinjection studies have not been performed on this organism, but fluoride, a G-protein agonist, can elicit GVBD at micromolar concentrations (Eckberg, unpub.). While this evidence is weak by itself, there is evidence that products of G-protein-mediated signaling systems regulate GVBD in this species (see below).

Cyclic AMP

Cyclic AMP has been implicated in the maintenance of oocytes in meiotic arrest (reviewed by Maller and Krebs, 1980; Masui, 1985; Sadler and Maller, 1985; Maller, 1988). Amphibian oocytes show a temporary decrease in intracellular cAMP upon hormonal stimulation (Maller and Krebs, 1977; Speaker and Butcher, 1977; Sadler and Maller, 1985; Cicirelli and Smith, 1985; Maller, 1987). Furthermore, microinjection of the regulatory subunit of A-kinase promotes maturation, whereas microinjection of the catalytic subunit of the enzyme inhibits it (Maller and Krebs, 1977). Therefore, it is reasonable to suggest that progesterone elicits GVBD by inhibiting adenylate cyclase (Fig. 3). Recently, the evidence for such a model in *Xenopus* has been reviewed (Sadler and Maller, 1985; Maller, 1988). Nevertheless, it is not clear that the decrease in cAMP is sufficient to elicit GVBD in frog oocytes. Early reports indicated a large decrease in intracellular cAMP after progesterone addition (Maller and Krebs, 1977; Speaker and Butcher, 1977), but it is not clear how inhibition of adenylate cyclase can cause a large rapid decrease in the cellular cAMP content. Recently, the magnitude of the decrease has been disputed (Cicirelli and Smith, 1985). These last data indicate that a significant decrease in oocyte cAMP content may not be an obligatory step in the induction of GVBD by progesterone. Furthermore, although no data contradict such an interpretation, it has not been demonstrated definitively that a change in the cAMP concentration of the magnitude observed after progesterone treatment is sufficient to elicit GVBD.

In starfish, cAMP apparently *can* regulate GVBD, but it is unclear whether it does so *in vivo.* Microinjection of the catalytic subunit of adenylate cyclase may (Dorée *et al.,* 1981) or may not (Mazzei *et al.,* 1981) block 1-MA-induced GVBD, but microinjection of the regulatory subunit has no effect (Mazzei *et al.,* 1981; Dorée *et al.,* 1981). Initial investigations indicated that the oocyte cAMP content does not change in response to 1-MA treatment (Mazzei *et al.,* 1981; Dorée *et al.,* 1981), although recent studies indicate a gradual 10–30% decrease in the cellular cAMP content (Meijer and Zarutskie, 1987). However, it is unclear whether a decrease of this magnitude is a sufficient signal for GVBD, because treatment of the oocytes with forskolin, resulting in a 35-fold increase in the cellular cAMP content, does not block GVBD (Meijer and Zarutskie, 1987). In some other species, including *Spisula* and *Chaetopterus,* chemicals that elevate the cytoplasmic concentration of cAMP can inhibit GVBD (Sato *et al.,* 1985; Eckberg and Carroll, 1987), but there is no evidence that cAMP regulates GVBD *in vivo.* Similarly, CTX can block the induction of GVBD in *Xenopus* oocytes by insulin and *ras* proteins, agents which probably do not elicit GVBD by affecting intracellular cAMP levels.

To complicate this picture further, recent evidence indicates that an increase in the cellular cAMP content *causes* GVBD in the brittle star, *Amphiopholis kochii* (Yamashita, 1988). This conclusion is based on the observations that chemicals that increase the cellular content of cAMP elicit GVBD and that the cellular cAMP content correlates positively with GVBD. Together these data demonstrate that intracellular levels of cAMP can regulate GVBD, but their involvement in the natural induction of GVBD varies between species.

Inositol Lipid Hydrolysis

Certain G-proteins transduce extracellular signals to the cytoplasm by activating a phosphatidylinositol-specific phospholipase C (Rodbell, 1980). This enzyme appears to exist in a membrane-bound (Cockroft *et al.,* 1984) and a soluble (Wilson *et al.,* 1984) form. The membrane-bound form is apparently regulated by a G-protein (Cockroft and Gomperts, 1985), whereas the soluble form is not. Both are Ca^{2+}-dependent and both hydrolyze phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) preferentially over phosphatidylinositol (PtdIns) at low free Ca^{2+} concentrations (Cockroft *et al.,* 1984; Wilson *et al.,* 1984). The membrane-bound form is apparently incapable of hydrolyzing PtdIns at physiological Ca^{2+} concentrations (Cockroft *et al.,* 1984).

Hydrolysis of all inositol lipids by phospholipase C results in the production of a membrane-bound second messenger, diacylglycerol, while the hydrolysis of each different inositol lipid also results in the production of a different water-soluble product, some of which have second messenger activity (reviewed by Nishizuka, 1984; Berridge and Irvine, 1984). Hydrolysis of $PtdInsP_2$ results in the production of inositol 1,4,5-trisphosphate ($InsP_3$), hydrolysis of phosphatidylinositol 4-phosphate (PtdInsP) results in production of inositol 1,4-bisphosphate ($InsP_2$), and hydrolysis of PtdIns results in production of inositol 1-phosphate (InsP). Of these, only $InsP_3$ has second messenger activity.

The most direct evidence for the involvement of inositol lipid hydrolysis in GVBD has been obtained in *Spisula* (Eckberg and Szuts, 1987; Bloom *et al.,* submitted) and *Xenopus* (Lacal *et al.,* 1987) oocytes. In *Spisula,* fertilization results in a loss of 25–35% of the radioactivity from both PtdInsP and $PtdInsP_2$ within 30 seconds after insemination. Thereafter, their specific radioactivities return to the preinsemination levels. These results clearly demonstrate that inositol phospholipid hydrolysis is a very early event after the fertilization of *Spisula* oocytes. Evidence that the products of this hydrolysis cause GVBD are presented below.

In *Xenopus* oocytes, inositol lipid hydrolysis follows the microinjection of transforming *ras* proteins (Lacal *et al.,* 1987). Microinjected *ras* proteins cause a significant disappearance of PtdIns and a significant production of InsP and diacylglycerol after a delay of a few minutes, with very little loss of PtdInsP and $PtdInsP_2$ or production of $InsP_3$ or $InsP_2$ (Lacal *et al.,* 1987). Since the earliest time point examined in this study was 2 min after *ras* protein microinjection, any rapid transient hydrolysis of $PtdInsP_2$ or PtdInsP comparable to that observed in *Spisula* would have been missed. Nevertheless, no diacylglycerol accumulated during the first two minutes, suggesting that there had been no hydrolysis of any inositol phospholipid during this time.

By contrast, PtdInsP and/or $PtdInsP_2$ hydrolysis appears to be necessary for insulin-induced GVBD because neomycin, a substrate-directed antagonist of phospholipase C, inhibits the response (Stith and Maller, 1987). Neomycin binds preferentially to $PtdInsP_2$ and to PtdInsP, but has little binding capacity for PtdIns (Schacht, 1978; Whitaker and Aitchison, 1985). Neomycin inhibition of insulin-induced GVBD would thus implicate PtdInsP and $PtdInsP_2$ hydrolysis. Together these results implicate inositol lipid hydrolysis in *Xenopus* GVBD, whether induced by insulin or by *ras* proteins. The apparent involvement of polyphosphatidylinositol hydrolysis in insulin-induced GVBD is somewhat surprising, because it suggests that the IGFR may be linked to a G_o-like protein. If this interpretation is correct, it should be confirmed by direct measurement of inositol lipid pools after exposure of the oocytes to insulin. Although fertilized *Spisula* oocytes and insulin-treated and *ras*-microinjected *Xenopus* oocytes all apparently use inositol lipid hydrolysis to elicit GVBD, the specific substrates preferred by phospholipase C in this response differ among them.

In *Chaetopterus,* there is only indirect evidence for this signaling mechanism in GVBD. Neomycin can block GVBD (Eckberg and Carroll, 1986). In other organisms, this pathway evidently has not been studied.

Inositol Trisphosphate and Calcium Release

Inositol 1,4,5-trisphosphate causes a direct release of Ca^{2+} from intracellular stores (Berridge and Irvine, 1984; Busa *et al.*, 1985; Clapper and Lee, 1985). $InsP_3$ can elicit responses from eggs. Sea urchin (Whitaker and Irvine, 1984; Turner *et al.*, 1986), frog (Busa *et al.*, 1985; Picard *et al.*, 1985a), and starfish (Picard *et al.*, 1985a) eggs all undergo a cortical reaction in response to $InsP_3$ microinjection.

In the starfish $InsP_3$ may not be involved in GVBD, because microinjected $InsP_3$ does not cause GVBD or prevent the oocyte from responding to 1-MA (Picard *et al.*, 1985a). Furthermore, despite many early observations which indicated involvement of Ca^{2+} release in the regulation of GVBD (see Meijer and Guerrier, 1985; Moreau *et al.*, 1985, for review), recent evidence indicates that Ca^{2+} transients are not required for 1-MA induced GVBD (Picard and Dorée, 1983; Eisen and Reynolds, 1984).

Similarly, microinjected $InsP_3$ does not elicit GVBD in *Xenopus* oocytes (Busa *et al.*, 1985; Picard *et al.*, 1985a). Nevertheless, microinjected $InsP_3$ can accelerate progesterone- or insulin-induced GVBD in *Xenopus* (Stith and Maller, 1987). If the effects of $InsP_3$ in this system result from Ca^{2+} release as expected, these observations indicate that elevated extracellular Ca^{2+} can *facilitate* GVBD, but is *not a primary signal* for it. Similarly, contrary to early reports (Moreau *et al.*, 1980; Wasserman *et al.*, 1980), a Ca^{2+} transient does not appear to be obligatory in the initiation of GVBD by progesterone (Robinson, 1985; Cork *et al.*, 1987). The cortical endoplasmic reticulum believed to be the source of the Ca^{2+} released at fertilization (Busa *et al.*, 1985) does not appear in oocytes until several hours after progesterone treatment (Charboneau and Grey, 1984). Thus, it is not surprising that $InsP_3$ does not elicit GVBD. The facilitating effect reported by Stith and Maller (1987) may be the result of a limited Ca^{2+} release from a rudimentary form of the cortical ER.

Spisula oocytes, in contrast to frog and starfish oocytes, undergo GVBD rapidly in response to microinjected $InsP_3$ (Bloom *et al.*, submitted). The effective concentrations (100% GVBD at a final intracellular $InsP_3$ of 60–80 n*M*) are very low and almost certainly must be exceeded as a result of the $PtdInsP_2$ hydrolysis that follows fertilization (Eckberg and Szuts, 1987; Bloom *et al.*, submitted). The results obtained clearly indicate that $InsP_3$ can elicit GVBD directly in this species. The discrepancy between these results and those obtained using frog and starfish oocytes are easily explained if one realizes that GVBD is triggered by fertilization in *Spisula,* and thus is a part of the egg activation process—a process that requires a Ca^{2+} flux in this and possibly all other species. Additionally, it must be remembered that *Spisula* oocytes undergo GVBD very quickly after fertilization (100% GVBD in >10 min). This rapid response may indicate that *Spisula* oocytes use signals not necessary in these other organisms.

Calmodulin

Calmodulin (CaM) is a small, highly conserved acidic protein that is involved in mediating the effects of Ca^{2+} on a number of enzymes (reviewed by Cheung, 1980). It is believed that CaM is present in excess in all cells and that it modulates cellular behavior by binding Ca^{2+} and then activating enzymes as a Ca^{2+}-CaM complex. Possible CaM involvement in regulating GVBD has been reviewed in more detail elsewhere (Meijer and Wallace, 1985).

CaM is present in oocytes, representing 0.2–0.5% of the cellular protein. It has been purified from frog (Cartaud *et al.*, 1980; Wasserman and Smith, 1981), starfish (Meijer and Guerrier, 1981), annelid (Carroll and Eckberg, 1983), and mammalian (Bornslaeger *et al.*, 1984) oocytes. CaM might promote GVBD by activating cyclic nucleotide phosphodiesterase. A CaM-dependent cyclic nucleotide phosphodiesterase has been reported in frog (Miot and Erneux, 1982) and mouse (Bornslaeger *et al.*, 1984) oocytes. Alternatively, CaM could elicit GVBD by activating a multifunctional CaM-dependent protein kinase (Stull *et al.*, 1986) (Fig. 2). Such enzymes have been demonstrated in frog (Wasserman and Smith, 1981) and starfish (Meijer and Wallace, 1985) oocytes. The existence of two reasonable mechanisms whereby CaM could regulate GVBD has made this protein an attractive system for study.

Several laboratories have studied possible CaM involvement in GVBD. These studies have followed two courses—microinjection of Ca^{2+}-CaM and microinjection or external application of CaM antagonists. Studies of the effects of microinjected Ca^{2+}-CaM on starfish oocytes have provided negative results (Dorée *et al.*, 1981; Meijer and Guerrier, 1981). In these studies, CaM neither inhibited nor facilitated GVBD when induced by 1-MA. By contrast, in frog oocytes microinjected Ca^{2+}-

CaM was reported to induce GVBD (Maller and Krebs, 1980; Wasserman and Smith, 1981). However, more recent studies were unable to confirm this finding (Cicirelli and Smith, 1987). The ineffectiveness of microinjected Ca^{2+}-CaM at inducing GVBD strongly suggests that CaM activation is not sufficient to elicit GVBD.

Antagonist studies, on the other hand, have provided evidence that CaM is necessary for GVBD in starfish (Meijer and Guerrier, 1981; Dorée *et al.*, 1982), *Chaetopterus* (Carroll and Eckberg, 1983), mouse (Bornslaeger *et al.*, 1984), and *Spisula* oocytes (Carroll and Eckberg, 1986). In frog oocytes, CaM antagonists evidently have different effects when they are applied externally than when they are microinjected (Cartaud *et al.*, 1980; Hollinger and Alvarez, 1982; 1984). Analogous results in starfish (Dorée *et al.*, 1981, 1982; Meijer *et al.*, 1981) oocytes led to the hypothesis that CaM is involved in the transduction of the stimulus for GVBD (Dorée *et al.*, 1982) or in some other early membrane-associated event. Such an hypothesis is also consistent with the observation that CaM antagonists only inhibit *Spisula* GVBD when applied within the first minute after fertilization, although Ca^{2+} is required for a longer time period (Carroll and Eckberg, 1986).

Several problems arise in trying to demonstrate a role for CaM in GVBD conclusively. First, CaM is activated by binding Ca^{2+}, and the evidence for a Ca^{2+} increase after induction of GVBD is controversial, as described above. Second, CaM is generally considered to be a soluble protein and has not yet been directly linked to any membrane-bound signal transduction mechanisms, so it is unclear how CaM could be involved in such transduction, although it could be involved in cytoplasmic amplification of transduced signals. Third, CaM antagonists are all known to affect other processes not dependent on CaM (see articles in Hidaka and Hartshorne, 1985), although some of the antagonists are more selective than others (*e.g.*, Mazzei *et al.*, 1984). In fact, evidence has been presented that certain CaM antagonists can block GVBD by a mechanism which is probably unrelated to CaM inhibition (Carroll and Eckberg, 1983). Fourth, the lack of a method for specifically activating CaM (other than by eliciting a cytoplasmic Ca^{2+} increase) makes it impossible to test directly whether CaM activation can cause GVBD. Thus, CaM may be involved in regulating GVBD, but if it is, the mechanisms involved are unclear.

Diacylglycerol and C-kinase

Inositol lipid hydrolysis by phospholipase C produces 1 mole of *sn*-1,2-diacylglycerol (DG) per mole of phospholipid hydrolyzed. Thus DG is the most abundant product of inositol lipid hydrolysis. DG has been identified as the physiological activator of C-kinase (see Nishizuka, 1984, for a review). Tumor promoting phorbol esters, such as 12-*0*-tetradecanoylphorbol 13-acetate (TPA), have also been shown to produce their cellular responses by binding to C-kinase and acting as a metabolically stable DG analog (Parker *et al.*, 1984; Tanaka *et al.*, 1986). A Na^+/H^+ antiport carrier is one substrate of C-kinase (Berridge and Irvine, 1984), and many of the effects of TPA on eggs can be explained as the result of activation of this enzyme and consequent cytoplasmic alkalinization (Swann and Whitaker, 1985; Lau *et al.*, 1986).

There is direct evidence that C-kinase activation necessarily precedes and can be sufficient to cause GVBD in *Spisula* oocytes (Dubé *et al.*, 1987; Eckberg *et al.*, 1987). The evidence includes several findings. First, TPA can elicit GVBD directly. Second, TPA elicits a pattern of protein phosphorylation similar or identical to that induced by fertilization. Third, C-kinase antagonists block GVBD whether induced by fertilization or by phorbol esters. Fourth, C-kinase activity can be demonstrated in oocyte extracts. Additionally Ca^{2+} and C-kinase apparently act synergistically in eliciting GVBD (Dubé *et al.*, 1987; Eckberg *et al.*, 1987). This also follows from the absolute Ca^{2+} dependence of *Spisula* GVBD and the ability of $InsP_3$ to elicit GVBD as well. Together these results strongly indicate that both products of inositol lipid hydrolysis act synergistically to elicit GVBD in this species (Bloom *et al.*, submitted). In this species, there is indirect evidence that the effects of TPA involve cytoplasmic alkalinization (Dubé, 1988).

In *Chaetopterus*, C-kinase activation appears to be both necessary and sufficient for GVBD (Eckberg and Carroll, 1987). However, the evidence for involvement of any specific kinase is indirect, because protein phosphorylation prior to GVBD has not been investigated. Phosphorylation precedes GVBD in another annelid, *Sabellaria* (Peaucellier *et al.*, 1982, 1984). Furthermore, the effects of TPA in this species do not appear to involve Na^+/H^+ exchange, because the effect is not dependent on extracellular Na^+ (Eckberg, unpub.).

Involvement of this pathway in GVBD does not appear to be restricted to invertebrates. TPA can elicit GVBD directly in frog oocytes (Stith and Maller, 1987). As with *Chaetopterus*, the effects of TPA do not depend on extracellular Na^+. Together with the lack of effect of $InsP_3$, these results suggest that DG is probably more important in regulating GVBD in the frog than the water-soluble products of inositol lipid hydrolysis. Furthermore, microinjected DG-activated C-kinase accelerates insulin induced GVBD, but has no effect on progesterone induced GVBD (Stith and Maller, 1987). These last results indicate that the metabolic pathways activated by C-kinase are also maximally activated by progesterone treatment, but not by insulin. Whether progesterone induced GVBD depends on the activation of C-kinase apparently has not been tested.

Whether other organisms use this pathway is uncertain. Phorbol esters can block spontaneous GVBD in mouse oocytes (Urner and Schorderet-Slatkine, 1984; Bornslaeger *et al.*, 1986), and they can elicit GVBD in follicle-enclosed rat oocytes (Aberdam and Dekel, 1985), but the physiological significance of these observations is unclear. The culture conditions required to prevent spontaneous GVBD in denuded mouse oocytes are probably unphysiological, and in follicle-enclosed rat oocytes the cellular site of action of C-kinase agonists is ambiguous.

The situation in starfish oocytes is similarly confusing. A G-protein apparently transduces the hormonal signal, but $InsP_3$ does not elicit GVBD, and the involvement of the oocyte cAMP content in GVBD is controversial (see above). Therefore, DG might be expected to cause GVBD by activating C-kinase. However, exogenously added C-kinase agonists do not elicit GVBD (Eckberg, unpub.). Moreover, phorbol esters can block 1-MA induced GVBD (Kishimoto *et al.*, 1985). The significance of this inhibition is not clear, however, because very high concentrations of phorbol esters were used. The effective doses for inhibition of 1-MA-induced GVBD by phorbol esters are about two orders of magnitude higher than either their dissociation constants for purified C-kinase or the doses affecting GVBD in other organisms. Since inositol lipid metabolism has not been studied in starfish oocytes, it is unknown whether inositol lipid hydrolysis follows hormone addition or if this process is necessary for GVBD. Thus, while the involvement of G-proteins in starfish GVBD is very probable, the exact nature of the involvement has not been sufficiently examined.

Phosphatases

Given the importance of protein phosphorylation in GVBD, phosphatase activity would be expected to modulate GVBD. Indeed, microinjection of specific phosphatases inhibits hormone induced GVBD in amphibian (Hermann *et al.*, 1984) and starfish (Meijer *et al.*, 1986) oocytes, and microinjection of phosphatase inhibitors can directly elicit GVBD in starfish oocytes (Pondaven and Meijer, 1986). Phosphatase inhibition of GVBD could be overcome either by microinjection of MPF or by increasing the extracellular hormone concentration. However, microinjection of phosphatase 1 min before GVBD, a time *after* MPF should have appeared in the cytoplasm, still blocks hormone-induced GVBD (Meijer *et al.*, 1986). This last observation is difficult to reconcile with the ineffectiveness of phosphatase at inhibiting GVBD when induced by microinjected MPF. Thus, these data do not allow us to specify the site in the sequence of events leading to GVBD at which phosphatases act. While these data do not conclusively demonstrate a mechanism for regulation of GVBD by phosphatases, they provide further support for the importance of protein phosphorylation in the regulation of GVBD.

Genes That Regulate Mitosis and Meiosis

Cyclins are proteins that are synthesized at an apparently constant rate during the cell cycle, but are abruptly destroyed during mitosis (Rosenthal *et al.*, 1980; 1983; Evans *et al.*, 1983). Cyclins also cycle during the meiotic divisions of *Spisula* (Swensen *et al.*, 1986) and starfish oocytes (Standart *et al.*, 1987). One of the *Spisula* cyclin genes (cyclin B) has a protein kinase consensus sequence (Westendorf and Ruderman, 1987), and cyclins A and B both have potential phosphate acceptor sites (Swensen *et al.*, 1986; Westendorf and Ruderman, 1987). Additionally, microinjected cloned mRNA for *Spisula* cyclin A can drive frog primary oocytes through GVBD to meiotic metaphase (Swensen *et al.*, 1986).

These data indicate that the synthesis if a *Spisula* cyclin can activate GVBD in frog oocytes. Whether cyclins have a role in GVBD in other organisms is unclear. Clam cyclin A does not appear at detectable levels until after GVBD (Swensen *et al.*, 1986), although cyclin B is detectable in the prophase-arrested oocyte (Westendorf and Ruderman, 1987). The possible involvement of starfish cyclin (only one such protein has been reported in these oocytes, and the mRNA for this protein is at least partially homologous with clam cyclin A mRNA) in GVBD is unclear, because it was not assayed until after GVBD. High levels of cyclin do not correlate directly with NEBD in these oocytes, however, because this protein accumulates in the oocyte after the formation of the second polar body, even though the oocytes remain arrested in interphase until fertilization (Standart *et al.*, 1987). Similarly, the degradation of cyclins does not drive the nuclei into an interphase configuration because cyclins are degraded at both meiotic divisions, but no nuclear reformation occurs between the meiotic divisions (Longo and Anderson, 1970; Longo *et al.*, 1982).

The observation that high levels of cyclin do not necessarily correlate with NEBD in starfish and *Spisula* oocytes, even though cyclin mRNA can drive frog oocytes into M-phase, can be explained if cyclin protein must be posttranslationally modified to an active state. Frog oocytes have the ability to modify proteins appropriately, whereas the other oocytes may not. Since the cyclins have potential phosphate acceptor sites, it is reasonable to suggest that their activity could be regulated by phosphorylation. This interpretation predicts that the phosphorylation state of "active" cyclin present during the interphase/M-phase transition would differ from that of "inactive" cyclin present during interphase. It also predicts that frog oocytes must rapidly modify the newly synthesized cyclin. An alternative explanation, that cyclin synthesis *does* drive GVBD in frog oocytes, is consistent with the fact that frog oocytes must undergo protein synthesis prior to GVBD, whereas oocytes of other species need not (reviewed in Masui and Clarke, 1979; Masui, 1985). The synthesis and/or postranslational modification of a cyclin may thus drive GVBD. In these interpretations, cyclin metabolism might be a common factor in the regulation of mitotic and meiotic cell division.

If mechanisms regulating mitotic and meiotic cell division are similar, then studies of the factors that regulate mitotic division should also have relevance to the study of meiotic cell division. Recently, a series of yeast genes regulating the passage through G_2 in the cell cycle has been reported. These include *cdc2, wee1,* and *nim1,* each of which encodes a protein serine/threonine kinase. The *wee1*$^+$ protein is an inhibitor that delays entry into M until the cell has reached a certain size (Nurse, 1975). This protein is a 112 kDa protein kinase (Russell and Nurse, 1987a) which apparently inhibits the *cdc2*$^+$ protein, a 34 kDa protein kinase (Hindley and Phear, 1984; Russell and Nurse, 1986) by phosphorylating it (Simanis and Nurse, 1986). Similarly, the *wee1*$^+$ protein is inhibited by *nim1*$^+$, a 50 kDa protein kinase (Russell and Nurse, 1987b). Thus a protein phosphorylation cascade regulates mitotic cell division in which mitosis occurs because an inhibitor of cell division is inhibited.

The *cdc2*$^+$ protein is an activator of M phase, analogous to MPF. It can also be positively modulated by the protein product of the *cdc25*$^+$ gene. The action of this gene product is required in addition to that of *nim1*$^+$ for the cell to reach M-phase. If the *cdc25*$^+$ protein proves to be a kinase, then phosphorylation would both positively and negatively regulate mitosis.

Conclusions, Model, and Prospects

It is evident, then, that intracellular signaling mechanisms similar or analogous to those involved in the regulation of somatic cell division and behavior are involved in the initiation of GVBD. It is also clear that, although protein phosphorylation is necessary for GVBD and is involved in the production and action of MPF, one single model cannot yet account for all of the data. Instead, kinases appear to regulate GVBD differently in different species and under different conditions. Therefore, we propose a model in which several different kinases act to promote or inhibit MPF activity, similar to the yeast model presented above. In our model, MPF is the central feature (Fig. 4).

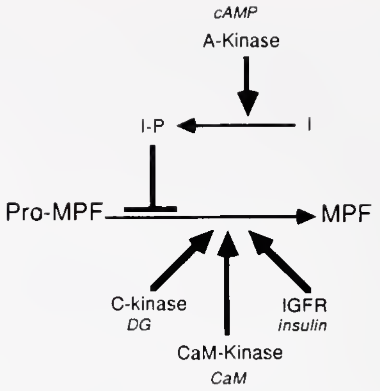

Figure 4. Proposed scheme for the interaction of kinases in the induction of GVBD. The cofactors for the active kinases are italicized. C-kinase, CaM-dependent kinase, and insulin-like growth factor (IGFR) each activates the conversion of Pro-MPF to MPF at a point downstream from the inhibitory effect of A-kinase. A scheme such as this allows the quantitative regulation of the conversion of Pro-MPF to MPF. Symbols are as in the legend to Figure 2.

Negative regulation of MPF is provided by A-kinase in most species. In principle, A-kinase could inhibit MPF activity either directly, by phosphorylating a site on MPF which inhibits its activity, or indirectly, either by activating an inhibitor of MPF or by inhibiting an activator of MPF. The evidence indicates that this antagonism of MPF is indirect (see above and Fig. 3). The activity of A-kinase as an MPF inhibitor is regulated in turn by the cellular concentration of cAMP. Thus a decrease in cellular cAMP antagonizes the inhibition of MPF activity (*cf.* Fig. 3). An analogy in yeast mitosis is the inhibition of *wee1*$^+$ protein by *nim1*$^+$ protein.

By contrast, many other kinases promote the activation of MPF. These include C-kinase, CaM-dependent protein kinase, IGFR, and pp60$^{v\text{-}src}$. These could activate MPF directly, they could activate one or more other protein kinases that directly activate MPF, or they could inactivate an inhibitor of MPF, such as A-kinase or an inhibitory phosphorylated substrate of A-kinase. Inactivation of A-kinase would not seem to be a reasonable explanation for the induction of GVBD by C-kinase in *Chaetopterus,* because C-kinase activators elicit GVBD even in the presence of very high concentrations of cAMP (Eckberg and Carroll, 1987). Similarly, the catalytic subunit of A-kinase does not prevent the amplification of MPF (Maller and Krebs, 1980), indicating that A-kinase is not a direct inhibitor of MPF. Thus we propose that protein kinase activities that elicit GVBD do so by activating MPF, either directly or indirectly, downstream from the inhibitory action of the A-kinase substrate (Fig. 4). Thus the intracellular activators and inhibitors of meiotic cell division interact in a protein phosphorylation cascade that can regulate GVBD both positively and negatively. The existence of coordinate and antagonistic protein kinase activities regulating GVBD would allow the oocyte maximum flexibility and sensitivity in responding to extracellular and intracellular signals regulating GVBD.

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An Assessment of Poecilogony in Marine Invertebrates: Phenomenon or Fantasy?

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Abstract. Poecilogony, defined as more than one mode of reproduction within a single species, has been reported in various invertebrates, including mollusks and polychaetes. Many cases that have been described involve planktotrophic and non-planktotrophic development in allopatric populations, or instances of planktonic larval stages and benthic juveniles being found together, but not associated with adults. There is always the possibility of mis-assignment of larvae to adults of the wrong species. Most cases that offer these kinds of evidence are now known to involve cryptic species, not poecilogony.

There are a few species in which release of young occurs both at metamorphosis and a day or so before. There may be cases in which extracellular yolk or nurse-egg production is variable and allows the release of larvae at different stages, but no actual instance is known. A few instances are known of allopatric populations with different modes of development and other differences in reproductive characters that lack reproductive isolation when brought into the laboratory. The polychaetes *Streblospio benedicti, Cirriformia tentaculata, Boccardia proboscidea,* and the opisthobranch *Elysia chlorotica* are in this category. All examples of poecilogony require further genetic substantiation.

Despite the scarcity of proven examples of poecilogony, the presence of more than one mode of reproduction within a genus is the rule in most invertebrate phyla. The evolutionary and ecological significance of these patterns is discussed.

Introduction

There have been numerous reports of more than one pathway of development within a single species of marine invertebrate. Quotes such as these are common: "It is an established fact that some animal forms have a different mode of development under different biological conditions" (Mortensen, 1921, p. 241). "Studies . . . cast further doubt on an already-failing dogma that a particular larval mode is a set and inflexible species characteristic" (Hadfield, 1972). The term poecilogony was invented to describe the phenomenon. ". . . poecilogony was first mentioned by Giard (1904) [sic], who gives a number of examples of its occurrence . . . which, as far as the echinoderms are concerned, have proved to be wrong. . . . That the ability exists is, however, a fact." (Thorson, 1950, pp. 29–30). If poecilogony exists, it is of interest to evolutionary biologists. However, further investigation has often revealed that cryptic species were responsible for the supposed poecilogony.

The word poecilogony derives from the Greek, *poikil-*, various, and *goneia*, reproduction. Even Webster's [unabridged] *Third New International Dictionary* (1971) expressed doubt as to whether poecilogony occurs: "A *supposed* method of development occurring in invertebrate animals when in the same species there are two kinds of young although the adults are *exactly* alike" (italics ours). In this paper, we first define the phenomena we mean to include under the term poecilogony. We then review reported cases of poecilogony known to us in marine invertebrates, particularly mollusks and polychaetes. We also demonstrate that congeneric species in many invertebrate groups differ in type of larval development, hence the mode of larval development has a complex history within most invertebrate phyla. The likelihood that poecilogony is real is assessed and theoretical implications are discussed. We suggest ways of testing the hypothesis of poecilogony in some cases where the data are insufficient for a judgment to be made.

Materials and Methods

We searched the published literature for references to poecilogony. We categorized the data in each paper as to

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the geographical or seasonal pattern found, the type of evidence for poecilogony, and the proposed mechanism. In some cases, we sought further information from the authors. We then evaluated the status of each claim of poecilogony.

Results

Definitions

Poecilogony is the presence of more than one distinctive kind of development in the same sexually reproducing species, *i.e.*, a polymorphism in development. In most cases, it involves differences in egg size (*e.g.*, small eggs destined to develop planktotrophically and large eggs that develop directly) or embryo size (*e.g.*, some embryos consume nurse eggs but others do not). It might occur as geographical variation between populations. In that case, it is difficult to know whether two species are involved. Some cases of poecilogony are cited as seasonal within a population. For example, a population produces brooded young in spring, but releases planktonic young in fall. A third reported kind of poecilogony is environmentally determined, as when the pattern of development is determined by salinity or amount of food available to the parent.

Giard (1891) first proposed the term "poecilogonie" in a short paper mainly discussing larval ascidians. He followed up with a paper devoted to poecilogony (1892), and then by a frequently cited lengthy article (1905). In this last paper, he said that poecilogonous differences could involve (1) certain individuals or different generations in one population (involving seasonal, nutritional factors, etc.), or (2) geographically separated populations with or without habitat differences. He believed that there are examples of poecilogony in nearly all the major groups of animals. Most if not all of these examples can be explained equally well as similar or sibling species with invariable larval development. Giard admitted that there can be differences, although slight, between different parents with different kinds of development.

In most of the proposed examples of poecilogony, observations have been made in the field, and the same individual parent or lineage could not be shown to produce two kinds of young. There have been observations of two kinds of larvae within a single egg case. If the egg cases were shown to be laid by one female without contamination and both types of larvae hatched, it would be a demonstration of poecilogony. If only the larger larvae hatch, it would be an example of cannibalism, not poecilogony.

Some marine invertebrates produce larvae that feed facultatively in the plankton (Chia, 1974; Perron and Carrier, 1981; Alatalo *et al.*, 1984; Kempf and Hadfield, 1985; Emlet, 1986). We do not consider facultative feeding to be true poecilogony because there are not two kinds of young with different developmental paths. There is no morphological differentiation in either larvae or supportive structures (egg capsule wall, etc.) that distinguishes two kinds. We also exclude cases of diapause, such as occur in crustaceans, and heterogeneous life cycles, such as occur in cnidarians and many polychaetes with both sexual and asexual life stages. Finally, nonpolymorphic, continuous variation in egg size, clutch size, or development time is not poecilogony.

This paper does not cover examples of poecilogony in freshwater invertebrates nor in plants ("amphicarpy"), where there are some well-proven examples.

A review of the literature

We searched the literature for reported cases of poecilogony. Jablonski and Lutz (1983) reviewed some molluscan examples, but most are scattered in the literature. Table I reports cases in the Mollusca; Table II gives cases in the Polychaeta. Most examples are in these two higher taxa. The kind of poecilogony is categorized as geographical (G) when reports of allopatric populations are involved, seasonal (S) when a succession of reproductive modes has been reported for a single population, sympatric (Sy) when occurring within a population simultaneously, capsular (Cp) when within a single capsule, and environmental (E) when factors such as salinity have been invoked to explain the occurrence of poecilogony. The type of evidence is also categorized: field observations of larvae without associated adults (F), field observations with adults (FA), production of young (L), or successful breeding of individuals differing in mode of reproduction in the laboratory (LB). If the different modes of reproduction were independent observations by two researchers, it is so indicated (I). If only embryonic shells or only planktonic veligers were observed, the symbols (Sh) and (P) are used, respectively. Mechanisms of poecilogony originally proposed are given: twinning (T), nurse-eggs (N), cannibalism (C), extracellular yolk (Y), and/or variable nutrition available to the parent (VN).

Table II reports cases in the Polychaeta, including the forms of reproduction reported: planktonic development (P), benthic development (B), brooding or ovoviviparity (Br), epitoky (Ep), atoky (A), and nurse-eggs (N), which are usually in benthic capsules.

Forty-two examples from the Mollusca have been found, all marine, eighteen of which involve geographically distinct populations (Table I). Nearly all prosobranch examples are of planktotrophic veligers and either direct development or demersal eggs. The family Littorinidae contains sympatric sibling species that differ in reproductive mode as well as penial morphology and other characters (Reid, 1986; Heller, 1975). Electrophoresis has confirmed in several cases the genetic distinctness of the sibling species (*e.g.*, Ward and Janson, 1985).

Reid (1986) places *L. angulifera* in the genus *Littoraria.* Although he has no specific evidence about the reproductive anatomy of this species, he concludes, "Anatomical examination has provided no suggestion that any species of *Littoraria* is able to show both oviparous and ovoviviparous modes of development." *Tectarius muricatus* has been reported by various workers in different localities to be "viviparous" (releasing fully developed veligers) or to release pelagic capsules, but differences exist in the radula that suggest several species could be involved (Bandel, 1974).

According to Rehfeldt (1968), Rasmussen suspected poecilogony for *Rissoa membranacea,* but she doubted it, based on her own data. Indeed, *R. membranacea* has been reported incorrectly synonymized with a morphologically distinct species, *R. labiosa* [Verduin, (1986a)]. Data from *Modulus modulus, Brachystomia* [*Odostomia*] *rissoides,* and *Natica groenlandica* (Table I) are probably also based on confusion of two allopatric congeners; there is no evidence of interbreeding of the different reproductive types. Risbec's (1935) brief description of *Siphonaria atra* is insufficient to know if two reproductive types exist. The case of *Siphonaria laciniosa* was disproved, being a case of mistaken synonymy (Hulings, 1986). Gallardo (1979) disproved his own example of *Crepidula dilatata* when morphological and ecological data were collected that revealed a sibling species. Likewise, the cephalaspid (bullomorph) genus *Acteocina* is now known to be composed of sibling species with different types of reproduction (Mikkelsen and Mikkelsen, 1984).

The species in Table I with egg collars or capsules that were collected without the parent, such as *Sipho islandicus,* the Columbellidae, and the Naticidae, provide poorly documented examples of poecilogony. The two types of *Columbella rustica* had different capsule morphology and therefore are most likely distinct species. There was no basis for the claim of poecilogony for *Lunatia pallida,* because two types of hatching young were not observed. The observation of a single egg mass with two embryos per capsule led to speculation that these young might be released as veligers instead of crawling young (Thorson, 1946). Similarly, the data for *Polinices catena* show some variation in numbers of nurse-eggs but poecilogony is only an extrapolation; positively identified *P. catena* with pelagic young have not been found (Thorson, 1946). Although two types of egg collars were found for *Polinices triseriata,* the association of the egg collars with particular adults was a guess by different investigators, and the oft-quoted correlation of larval type with local weather was only a suggestion based on collections of eggs made once each in two years (Giglioli, 1955).

The finding of two different types of protoconchs in planktonic juveniles cannot be taken as proof of poecilogony because of the great possibility of mis-assignment of the juveniles to adults. For example, Taylor (unpub.) reported finding veligers in the plankton that belonged to several species of Vermetidae previously known to produce crawling young. Her reports, cited in Hadfield *et al.* (1972), are undocumented. Verduin (1986b) reported that *Alvania cimex* was really a species complex. Thiriot-Quiévreux's (1980a) *Scila adamsi* var. *beauforti* with a shell distinct from *S. adamsi* is probably a distinct species.

Examples of poecilogony involving nurse-eggs include *Sipho islandicus, Planaxis sulcatus, Thais canaliculata,* and *T. haemastoma.* The last three are based on geographically isolated populations. The assumption that numbers of nurse-eggs vary to produce two hatching types within a single population or from a single adult is not based on any direct observations and is not justified by the data. Spight (1977) suggested that *Thais canaliculata* was evolving nurse-eggs, from his finding of egg capsules in separate localities with and without nurse-eggs. Only two capsules were found with adults; Spight did not compare the adults that produced the capsules. He found variation in percent of developing embryos in the populations with nurse-eggs. No evidence was presented that bears on whether one or two species exist. Rivest (1981) found nurse eggs in all populations that he examined from six localities, including the one studied by Spight. It is possible that Spight examined capsules at one site after all nurse eggs had been consumed.

"*Dolium maculatum*" [=*Tonna maculosa*] is interesting in that it is reported to have brood cannibalism, in which embryos of different size and developmental stage exist in the same capsule (Thorson, 1940). There is no proof that the less-developed young ever are released from the egg mass and survive, so we cannot say that there are two kinds of larvae. This situation may just be a variant in which nurse-eggs begin to develop, albeit slowly, before being consumed.

Penchaszadeh (1981) reported poecilogony in *Tonna galea.* He found small and large larval shells (reflecting, respectively, planktotrophy and lecithotrophy) in a single egg case. However, as can be seen even from his photographs (Penchaszadeh, 1981, Figs. 1a, b), the large larval shells are not shaped like larval *Tonna,* but have a much longer anterior siphonal canal. (See Laursen, 1981, pp. 31–34, Figs. 49 and 51 for illustrations of larval *Tonna* shells.) A growth series of ten larval specimens from the Atlantic Ocean, loaned to us by Dr. Rudolf S. Scheltema, corroborates Laursen's observations: at no stage does larval *Tonna* have a long anterior siphonal canal. Even though some similar observations on *Tonna* larvae were published earlier suggesting brood cannibalism (Thorson, 1940), there is at least one other explanation. Possibly some oophagous gastropod pierced some of the compartments, ate the contained *Tonna* eggs, and

Table I

Reported cases of poecilogony in Mollusca. Abbreviations are explained in the text. The classification follows Taylor and Sohl (1962) for prosobranchs and Thompson (1976) for opisthobranchs

Family	Genus & species	Citation	Type	Mechanism	Evidence	Comments; status
POLYPLACOPHORA						
Chitonidae	*Chiton cinereus*	**Thorson, 1946	G, Sy	—	FA, I	Demersal eggs or brooding in mantle cavity; same stage at hatching.
GASTROPODA						
Littorinidae	*Littorina angulifera*	*Lenderking, 1954	G	—	FA	Probably a species complex (Reid, 1986).
		**Mileikovsky, 1971	S	—	F, I	
	Littorina scabra	**Mileikovsky, 1975	G	—	FA, I	A species complex (Reid, 1986).
	Littorina scutulata	Imai, 1964	—	—	FA	Shown morphologically that 2 distinct species exist (Murray, 1982).
		*Buckland-Nicks *et al.*, 1973	G	—	FA, I	
	Littorina saxatilis	*James, 1968	G	—	FA	Species complex. See Heller, 1975; Ward & Janson, 1985; Hannaford Ellis, 1978, 1983. Claims of viviparity when young based on smaller species.
		Hughes, 1978	Sy	—	FA	
		*Bergerard & Sy	S	—	FA	
		Caugant, 1981	—	—	FA	
		Seshappa, 1948	Sy	—	—	
	Tectarius muricatus	*Bandel, 1974	G	—	F, I	Release of veligers *vs.* release of egg capsules
		Lebour, 1945	G	—	F, I	
Rissoidae	*Alvania cimex*	*Thiriot-Quiévreux, 1986b	Sy	—	Sh, P	Direct & pelagic development based on protoconch. Ornamentation differed. Verduin claimed complex of 3 species.
	Rissoa membranacea and	*Rehfeldt, 1968	—	—	—	Two species, based on morphology. Poecilogony doubted.
	Rissoa labiosa	Verduin, 1986a	—	—	—	
Vermetidae	*Dendropoma irregularis* (=*Spiroglyphus*)	*Lewis, 1960	?	—	FA	Some capsules produce briefly-planktonic young; others produce young that crawl away. Unclear if from same female or same locality.
	Dendropoma meroclista	**Hadfield *et al.*, 1972	Sy	—	P	Usually crawling young. Taylor (unpubl.) reported veligers in the plankton, same general locality.
	Dendropoma platypus	**Hadfield *et al.*, 1972	Sy	—	P	Same comment as just above.
	Petaloconchus keenae	**Hadfield *et al.*, 1972	Sy	—	P	Same comment as just above.
Planaxidae	*Planaxis sulcatus*	**Thorson, 1940, 1952	G	N	F, I	Nurse eggs in only some populations.
		Risbec, 1921	—	—	F	
		Lamy, 1928	—	—	F	
Modulidae	*Modulus modulus*	*Houbrick, 1980	G, E	—	L	Shell sculpture differences. Spawn masses differ in morphology.
		Lebour, 1945	—	—	F	
		Bandel, 1976	—	—	F	
Cerithiopsidae	*Seila adamsi*	*Thiriot-Quiévreux, 1980a	Sy	—	Sh, P	Protoconch differences. *S.a.* var. *beauforti* named.
Calyptraeidae	*Crepidula dilatata*	*Gallardo, 1977; 1979	—	—	FA	Two species, morphology and microhabitat differences. *C. fecunda* named.

Table I (Continued)

Family	Genus & species	Citation	Type	Mechanism	Evidence	Comments; status
Naticidae	*Lunatia pallida* (=*Natica*)	*Thorson, 1946	Sy	T	F	Speculation only; variation in egg size (Thorson, 1950).
	Natica groenlandica	*Thorson, 1950	G	—	F	Latitudinal differences.
	Polinices catena (=*Natica*)	#*Thorson, 1946	G	N	F, I	Number of nurse-eggs might vary. Thorson questioned Lebour's identity of *N. catena* with pelagic young.
		Ankel, 1930	—	—	F	
		Lebour, 1936	—	—	F	
	Polinices triseriata	*Giglioli, 1955	E	—	F, I	Different researchers, different years, same locality. Egg collars only. Wet vs. dry yrs.
		Stinson, 1946	—	—	F	
		Wheatley, 1947	—	—	F	
Tonnidae	*Tonna maculata*	*Thorson, 1940	Cp	C	F	"Brood cannibalism" cited.
	Tonna galea	*Penchaszadeh, 1981	Cp	C	F	1 egg mass fragment had 2 sizes of embryos.
Muricidae	***Murex incarnatus*	*Gohar & Eisawy, 1967	C	N	L	Embryological study. Most hatch as veligers & metamorphose next day; some hatch metamorphosing.
	Thais canaliculata	*Lyons & Spight, 1973;	G	N	FA, I	Yolky eggs at one locality, nurse eggs at another. % nurse eggs varies.
		Spight, 1977	G	N	F	
		Houston, 1971	—	—	FA	
	Thais haemastoma (confused with *T. floridana*; see Radwin & Chamberlin, 1973)	#*Thorson, 1950	G	N	F, I	Nurse eggs in some localities; error in report of nurse eggs in W. Indies (D'Asaro 1966; Spight, 1975).
		Lamy, 1928	—	—	F	
		Burkenroad, 1931	—	—	F	
Columbellidae	*Columbella rustica*	*Knudsen, 1950	E	—	F	Nurse eggs at Naples, Banyuls; veligers elsewhere. Capsules with nurse eggs a different shape; not collected with adults.
		*Thorson, 1950	G	N	F, I	
		Franc, 1943	—	—	F	
		Bacci, 1947	—	—	F	
		Bandel, 1975	—	N	F	
Buccinidae	*Sipho (Colus) islandicus*	*Thorson, 1935, 1936, 1952	Sy	N	F, L	Dredged field collection; adults apart from egg capsules in many cases. Assumed that young escape when nurse eggs depleted.
Pyramidellidae	*Brachystomia rissoides*	*Rasmussen, 1944, 1951	G, E	—	FA, L	Salinity proposed as controlling factor. No sympatry. No experimental evidence.
		Pelseneer, 1914	—	—	FA	
		Thorson, 1946	G	—	FA	
Acteocinidaea	*Acteocina candei & A. canaliculata*	*Franz, 1971	G	—	L, I	Two species (Mikkelsen, 1984).
		Wells & Wells, 1962	—	—	F	
Elysiidae	*Elysia cauze*	*Clark *et al.*, 1979	E, S	Y	FA	Sequential seasonal change, 3 types of development. No adult produced more than one type offspring.
	***Elysia chlorotica*	*West in Bonar, 1978	Cp	—	—	Lecithotrophic veligers & metamorphosing young.
		West *et al.*, 1984	G	—	LB	Hybridization between kinds.
	Elysia evelinae	*Clark & Jensen, 1981; Jensen & Clark, 1983	Cp	—	L	Capsular metamorphosis and lecithotrophy

Table I (Continued)

Family	Genus & species	Citation	Type	Mechanism	Evidence	Comments; status
Elysiidae (cont.)	*Tridachia crispata*	*Clark & Jensen, 1981	G	—	L	Capsular metamorphosis and lecithotrophy.
Dendronotidae	*Dendronotus frondosus*	*Clark, 1975	G	—	FA, I	Europe vs. New England populations.
		Thompson, 1967	—	—	FA	
Arminidae	*Armina tigrina*	*Eyster, 1981	G	—	L	Florida and S. Carolina. Based on egg diameters.
		Clark & Goetzfried, 1978	G	—	FA	
Spurillidae	*Spurilla neapolitana*	*Clark & Goetzfried, 1978	E	VN	L	Multi-embryo planktotrophic capsules produced when adults starved; otherwise nonplanktotrophic.
Cuthonidae	*Cuthona nana*	*Harris *et al.*, 1975, 1980	S	Y	F	Data from different years. Brown synonymized 2 species; Rivest (1978) found 2 species.
		Brown, 1978	G	—	F	
	**Tenellia fuscata* (=*Embletonia*) [see text for taxomony]	*Eyster, 1979	Sy	—	LB	Sympatric individuals bred in lab; offspring type didn't change. No proof of cross-fertilized matings. Morphological differences between larval types; adult size differed.
		Rasmussen, 1944	G, E	—	L	
Siphonariidae	*Siphonaria atra*	*Risbec, 1935	Sy	—	FA	Insufficient information.
	Siphonaria laciniosa *S. kurracheensis*	Hulings, 1986	—	—	—	Two species. Incorrect synonymy demonstrated.
BIVALVIA						
Teredinidae	*Teredo navalis*	*Kudinova-Pasternak, 1957; Thorson, 1946	G	—	F, I	Misidentifications of specimens in Adriatic Sea.
	Teredo utriculus	#*Kudinova-Pasternak, 1957;	S	—	F, I	Misidentifications; confused with *T. navalis.*
		Roch, 1940	—	—	F	
	Teredo pedicellata	#*Kudinova-Pasternak, 1957;	G	—	F, I	Probably confused with *T. navalis.*
		Roch, 1940	—	—	F	

* Primary reference suggesting poecilogony.

** Cases with merit.

# Author reported poecilogony on the basis of the research of others.

laid one of its own eggs in each compartment that it had emptied. To prove or disprove this idea, it should be possible to ascertain whether each compartment containing large larvae had been pierced prior to hatching.

There are many reports of poecilogony in nudibranchs and ascoglossans. As with all the foregoing examples, not one adult has been directly observed to produce more than a single distinct kind of offspring (but see discussion of *Elysia evelinae* and *E. chlorotica* below). The reported seasonal progression of three types of larvae for *Elysia cauze* (Clark *et al.*, 1979) could be the succession of three or even four species (Jensen and Clark, 1983). DeFreese and Clark (1983) report that sibling species account for two of the types of reproduction. Clark (pers. comm.) believes *E. cauze* to be a junior synonym of *E. subornata.* Allopatric populations of *Armina tigrina* and *Dendronotus frondosus* are undocumented as cases of poecilogony and need further systematic study; Clark (pers. comm.) points out that there are two available names for *Armina tigrina* in the southeastern United States, and there are differences in hatching morphology between the two allopatric forms, suggesting that two species exist. Poecilogony in *Tridachia crispata* is also insufficiently documented. From original published reports, it is not clear if capsular metamorphosis and lecithotrophy occurred in the same or different populations (Clark and Jensen, 1981). Clark (pers. comm.) stated that the populations are allopatric but within about 10 kilometers of each other; one is on a reef and the other is in mangroves. The two populations differ morphologically. Rivest (1978) demonstrated that the suggested poecilogony of *Cuthona nana* was in fact a case of sibling species.

There are a few remaining cases with better data. Specimens of *Tenellia pallida* of different reproductive modes were bred in the laboratory (Eyster, 1979). Offspring of the kind of the (female) parent were produced. There was no proof that cross-fertilization took place in these hermaphrodites, and morphological differences were found in the larvae of the two types. Genetic data on such a breeding (and on an F_2 generation) are badly needed to see if the forms are truly interfertile. Rasmussen (1944) found geographically separate populations presumed to be of this species, one that produced veliger larvae and another that produced veligers that metamorphosed as they hatched. Clark (pers. comm.) believes that Rasmussen's European populations are correctly called *Tenellia pallida*, while Eyster's American specimens are properly referred to *Tenellia fuscata*. For *T. fuscata* from New England, Harris *et al.* (1980) suggest not poecilogony, but a plasticity of hatching time determined in part by breakdown of the egg mass itself. Although the species is genetically programmed to go through a veliger stage, Harris *et al.* (1980) speculated that the exact time of hatching might be determined by bacteria, water movements, or other external factors.

Spurilla neapolitana was reported to produce a different kind of capsule when adults were starved (Clark and Goetzfried, 1978). This work should be continued to verify that the same adult could produce both kinds, and both kinds of capsule produced viable young. The original work involved adults in aquaria, not isolated individuals in finger bowls, and contamination could have occurred.

The best case for poecilogony in mollusks is that of *Elysia chlorotica*, in which hybridization of individuals from allopatric populations of different reproductive characters was achieved (West *et al.*, 1984). The F_1 generation produced young of the same type as the (female) parent, but the F_2 produced young that were intermediate in egg size and developmental characteristics, as if maternal expression were a factor. The interesting findings definitely call for further study. This case could represent genetically diverging (speciating) populations that have not yet become reproductively isolated, *i.e.*, true poecilogony.

Jensen and Clark (1983) and Clark (pers. comm.) report that capsular metamorphosis and lecithotrophy both occur in a gradient of developmental patterns within the same egg capsules in *Elysia evelinae*. The time between release from the capsule and metamorphosis of lecithotrophic young varies over a period of a day or two. The same situation occurs in the nudibranch *Doto acuta* (Schmekel and Kress, 1977), in *Murex incarnatus*, and in one of the two kinds of *Elysia chlorotica*: most young hatch as very short-term veligers, but a few larvae are retained in the capsules longer and hatch metamorphosing (Gohar and Eisawy, 1967). Such a situation could be considered incipient poecilogony, but there is no difference between the young in larval morphology or egg capsule morphology, and metamorphosis merely varies within a 24-hour period.

Williams (1980) reported two types of larvae for both *Hermissenda crassicornis* and *Aeolidia papillosa*. All eggs were the same size and all larvae were planktotrophic, but some hatched with yolk reserves and others hatched without yolk. The yolk-free larvae tended to be larger, and it was suggested that they developed faster. This situation does not quite fit the definition of poecilogony because there are not two distinct types of larvae, but it is a possible intermediate of the type needed to explain evolution of different reproductive modes.

Examples of poecilogonous bivalves (end of Table I) are limited to the genus *Teredo* of the Teredinidae. These boring bivalves are difficult to identify without dissection from the wood. Claims of poecilogony are unsubstantiated in the 30 years since Kudinova-Pasternak's 1957 paper, which was based not on laboratory anatomical comparisons or breeding studies but on various field reports. *Teredo utriculus* (=*T. norvagicus*) is oviparous and *Teredo pedicellatus* is larviparous, but both have been confused with *T. navalis* (briefly larviparous, then planktonic development ensues) (Turner, pers. comm.). Turner has found cryptic species pairs with different types of reproduction; *T. pedicellatus* is a species complex.

The case for *Chiton cinereus* is based on observations of eggs retained in the space surrounding the gills. Thorson (1946) was not sure if this retention was fortuitous or a regular occurrence; in any event, the stage at hatching does not change.

In the 22 examples of polychaetes (Table II), most cases clearly involve cryptic species in which the nonreproductive adults look alike, but the juvenile stages and/or reproductive adult stages differ. Often (*e.g.*, Thorson, 1946), researchers found larvae in the plankton and made an educated guess as to which species they belonged. Brooded larvae, of course, and most epitokes were associated with adult females.

Many supposed cases of poecilogony are found in the Spionidae, a group of small worms that can undergo copulation or produce spermatophores (Rice, 1978) and often brood young or have nurse eggs (Woodwick, 1960). *Spio setosa*, *S. martinensis*, and *Polydora quadrilobata* involve cryptic species and failure of researchers to associate larvae with the proper adults. Larvae of the benthic and planktonic types differ in numbers of setigers, setal pattern, color pattern, and ciliation, characters that are of taxonomic importance. *Spio filicornis* was misidentified and wrongly synonymized with *S. martinensis* (see Hannerz, 1956). Hannerz (1956) also disproved seasonal poecilogony for *Pygospio elegans*, finding only one morphology, but the possibility remains that the relative lengths of brooded and pelagic stages vary seasonally. However, Anger (1984) found in laboratory studies that temperature and salinity changes do not cause changes in developmental pattern of *P. elegans*. Thorson's (1946) three types of pelagic larvae of *P. elegans* differed in color, bristle lengths, etc. and obviously were different species.

Boccardia proboscidea has been confused with another species, *B. columbiana* (Woodwick, 1963). However, Woodwick (1977) found sympatric populations of *B. proboscidea* with lecithotrophic and pelagic larvae at Moss Landing, California. The two types of larvae differed in length of setae at the three-setiger stage. Not only were the eggs of lecithotrophically developing worms larger, but the capsules and three-setiger larvae were also larger. Therefore, the reproductive differences involve more than simple availability of nutrients. Hartman's (1941) descriptions of reproduction in this species dealt mainly but not exclusively with pelagic larval forms; all her adult specimens came from hard substrata. Woodwick, on the other hand, collected his specimens in sand and soft mud; his populations were mostly lecithotrophic. Whether interbreeding occurs in nature between the adults of the two different larval types could be assessed easily by comparing allozyme frequencies of sympatric individuals.

Blake and Kudenov (1981) reported *Boccardia proboscidea* from Australia with single egg capsules containing at the same time unfertilized nurse eggs, small larvae, and precocious larvae feeding on both the eggs and small larvae. They believed that the small larvae could have a long pelagic period if they survived brood cannibalism. However, none were observed to be released into the environment. If they were artificially removed from capsules in the laboratory, the small larvae could feed on suspended food particles (Blake and Kudenov, 1981). However, evidence that both types of larvae survive and contribute to future generations is needed. Production of developing embryos of two distinct size classes in one adult demonstrates that two modes of oogenesis can evolve in spionids. This is a most interesting population for further study of evolution of life history, and could be an example of true poecilogony. The Australian population with two sizes of larvae in the same capsule is believed to be a recent introduction (Blake and Kudenov, 1981).

In addition to *Boccardia proboscidea,* four other species in the genus have been reported by Read (1975) to reproduce with or without nurse eggs. He did not say whether two types of reproduction occurred in the same locality. He listed all localities together in a "material examined" section, apart from the reproductive data. Although he stated, "Some polydorids vary the form of larval development . . . by alteration of the amount of food supplied", no data were given. For *B. acus,* specimens from Wellington were larger, with more segments, branchiae, and setae, than specimens from Otago (New Zealand). It would be interesting to know if these localities represented the two reproductive types. If so, we predict that the Wellington specimens are a different species, with pelagic development.

Streblospio benedicti and *Cirriformia tentaculata* are the most interesting examples in the Polychaeta because breeding data are available. Levin (1984) crossed allopatric adults of two larval types of *S. benedicti,* planktotrophic and lecithotrophic. Inter-fertility was indicated. Further studies using sympatric specimens of the two reproductive types from Bogue Sound, North Carolina, indicated that F_1 and F_2 offspring were intermediate between the two parental types in egg size, brood size, and planktonic period (Levin and Creed, 1987, unpub. abstract, Second International Polychaete Conference). Hybrid individuals are sometimes observed in the field. Genetic data in the form of allozyme studies of field populations would be of high interest, to see if the two developmental types are fixed for alternative alleles at any locus in the sympatric population. If so, they are not breeding in nature and are functionally separate species. If not, they may well be a single polymorphic species, but the mechanism for maintaining such a polymorphism is difficult to imagine. Genetic studies of the laboratory-reared specimens would confirm whether cross-fertilization did occur in the breeding experiments. It is interesting that morphological differences between the two kinds of *S. benedicti* do occur: planktotrophic larvae have long swimming setae lacking in the non-feeding larvae, yolk in the two forms differs in composition, and ovaries first occur in setigers 9–11 of the planktotrophic females but in setigers 11–14 of the lecithotrophic females. "Females with planktotrophic development were longer, had more segments, bore the first gametogenic segment more anteriorly, had half the ovum diameter, . . . [and] more paired brood pouches . . . [than] those with lecithotrophic development." (Levin and Creed, 1986).

The initial hypothesis that *S. benedicti* is an example of incipient speciation between Atlantic and Pacific North American populations, with interbreeding still possible, was weakened by Levin's find of sympatric specimens in North Carolina. However, in some months planktotrophic reproduction only was found, suggesting that the two reproductive types were possibly partially segregated by maturing at different times. Animals reared under a variety of food levels and temperature regimes mimicking fall, summer-fall, and winter-spring did not change reproductive mode (Levin and Creed, 1986), so it is unlikely that the same individuals vary their reproduction seasonally. The genetic structure of *S. benedicti* cannot allow free interbreeding of the forms, or there would be a full range of forms rather than only occasional intermediates.

Coincidentally, Levin (1984) found a third type of *Streblospio benedicti* in the Gulf of Mexico that differed in adult reproductive structures; in particular, it had vascularized branchiae instead of brood pouches. It may represent a distinct species (Levin, pers. comm.).

George (1967) bred *Cirriformia tentaculata* from Plymouth, England, by mixing eggs of one female with male gametes in the laboratory. The gametes were naturally released. He reared both free-swimming and demersal larvae from single broods. No morphological differences were reported in the larvae. There was no detailed description of the methods of rearing so contamination cannot be ruled out, but the data certainly warrant further examination of *C. tentaculata.* Some populations are reported to be demersal only, with smaller adults (George, 1963), suggesting that two species could be involved. Grassle and Grassle (1976) showed the power of careful morphological study and electrophoresis when they found that differences in reproduction in *Capitella capitata* actually involved at least six sympatric species. Healy and Wells (1959) found new taxonomic characters that divide *Arenicola claparedei* into three species.

Claims made for poecilogony in geographically separated populations of polychaetes such as those of *Eupolymnia nebulosa* from the Mediterranean and the Atlantic coasts of France are without proof unless breeding studies have been done. Daly (1972) described brooding in *Harmothoe imbricata* outside Greenland, whereas Thorson (1936) found brooding in Greenland and pelagic development elsewhere, but no sympatric populations with two types of reproduction have been reported. Cases of poecilogony where adults differ consistently in size (incorrectly assumed to be age-related) are suspect; they include *Syllis vivipara* and *Ehlersia nepiotoca* (Table II).

Although Rasmussen (1956) found sympatric reproductive differences in *Nereis pelagica,* one of his adult types was a tube-builder and the other was not, indicating that he had two species distinct in microhabitat. *Nereis* (=*Platynereis*) *dumerilii* needs further examination because sympatric populations were found (Thorson, 1936). Simple allozyme studies might easily resolve the issue. Hempelmann's (1911) observation of two allopatric reproductive types was based on his synonymy of *Platynereis massiliensis* (Moquin-Tandon, 1869) with *P. dumerilii.* The former is atokous and produces benthic young in tubes protected by the male; the latter produces pelagic young via epitoky. Hauenschild (1951) showed that these are different species, on the basis of sperm morphology as well as on larval and adult behavioral characters that prevent cross-fertilization. Likewise, *P. megalops* (Verrill) of the North American Atlantic has been synonymized with *P. dumerilii* of Europe, but Smith (1958) showed that reproductive behavior (sexual dimorphism of heteronereids and copulation of *P. megalops* prior to release of eggs) prohibits interbreeding between the two species under natural conditions. Martin (1933) rejected poecilogony in favor of an interpretation of a complex of species for *Dodecaceria concharum;* there could be at least three species (Clark, 1977).

The phenomenon of embryos feeding on non-developing eggs or on other embryos is widespread in prosobranch gastropods and in spionid polychaetes, in which the term adelphophagy is often used (Mileikovsky, 1971). In the gastropods *Thais emarginata, Crepidula cerithicola,* and *Searlesia dira,* embryos from a single brood hatch at different sizes depending on the number of nurse eggs consumed (Spight, 1976; Rivest, 1983; Hoagland, 1986). However, there was only one stage at hatching for each species, despite size differences.

Very few examples of poecilogony have been given for higher taxa other than mollusks and polychaetes. Giard's (1891, 1892, "1904" [1905]) original examples included ascidians, coelenterates, crustaceans, flatworms, and many other groups; none of his cases were documented. Mortensen (1921) disproved the echinoderm examples. In the crustaceans, variation in egg size is fairly common, *e.g.,* in *Palaemonetes varians* (Boas, "1889" [1890]) and *Crangon crangon* (Thorson, 1946). However, we found no documented examples of poecilogony. The closest possibility is *Balanus balanoides,* reported to release naupliae ordinarily, but cypris-stage larvae in special circumstances (Runnström, 1925). Cryptic species cannot be ruled out. Most of the smaller phyla are too poorly known to provide data on poecilogony.

Several species of parasitic nematodes in at least three genera produce two types of eggs: thick-coated ones that pass to the exterior and thin-coated ones that go through an endogenous cycle (Adamson, 1984). These species also have haplodiploidy as a means of sex determination. These phenomena have been called poecilogony. No similar phenomena are known for marine invertebrates. Rotifers, freshwater sponges, copepods and other freshwater invertebrates do have resting eggs as a part of complex life cycles.

While documented cases of poecilogony are few to none, depending on the rigor of proof one is willing to accept, there are many genera in numerous phyla that have more than one mode of larval development (Giese and Pearse, 1974; 1975a, b). A complete list would be very long indeed. Radwin and Chamberlin (1973) summarized data for the Recent stenoglossan neogastropods. The few families whose living representatives apparently lack planktonic larval stages are the Buccinidae, Melongenidae, Fasciolariidae, Volutidae, Marginellidae, and Cancellariidae. Most other families of meso- and neogastropods contain genera with more than one type of development; these include the Littorinidae, Rissoidae, Cerithiidae, Hipponicidae, Calyptraeidae, Cypraeidae, Naticidae, Muricidae, Thaididae, Columbellidae,

Table II

Reported poecilogony in polychaetes. Asterisks as in Table I

Family	Genus & species	Citation	Type	Larval forms	Evidence	Comments; status
Polynoidae	*Harmothoe imbricata*	*Thorson, 1936, 1946	G	Br, P	FA	Eggs beneath elytra in Greenland, pelagic elsewhere.
		Izuka, 1912	—	P	FA	
Syllidae	*Syllis vivipara* & *S. prolifera*	**Thorson, 1950	Sy	Br, P	—	Synonymized by Thorson. Smaller are viviparous.
		Mesnil & Caullery, 1917	—	Br, P	F	
	Ehlersia nepiotoca & *E. sexoculata*	**Thorson, 1950	Sy	Br, P	—	Synonymized by Thorson. Smaller are viviparous.
		Mesnil & Caullery, 1917	—	Br, P	F	
Nereidae	*Nereis* (=*Ceratonereis*) *costae*	*Durchon, 1957	G	Ep, B	F	Epitokous form not studied. Atokes live in tubes.
	Nereis pelagica	*Rasmussen, 1956	Sy	B, P	F, L	Rasmussen's adults differed in microhabitat.
		Herpin, 1925	G	B	L, I	
		Wilson, 1932	G	P	L, I	
	Nereis zonata	*Thorson, 1950	G	Ep, B	FA, L	Epitokes or non-pelagic eggs, inner *vs.* outer fjords.
	Platynereis dumerilii	*Thorson, 1936	Sy	Ep, P	FA	Epitokes and non-pelagic eggs at Naples.
		Hempelmann, 1911	G	—	F	
		Wistinghausen, 1891	G	—	F	
	Perinereis cultrifera	*Durchon, 1957	G, Sy	A, Ep	F	Major life history differences between atokous and epitokous forms. Sympatry is relatively rare.
Onuphidae	*Diopatra cuprea*	*Monro, 1924	G	Br, P	F	Brooding in Panama.
Spionidae	*Polydora quadrilobata*	*Blake, 1969	G	N, P	F	Middle population differs from the two geographic extremes. Larvae differ morphologically.
	Spio filicornis	*Thorson, 1936	S	N, P	F	Nurse eggs in autumn. Söderström's (1920) incorrect synonomy with *S. martinensis* confused larval types (Hannerz, 1956).
		*Mesnil & Caullery, 1917	—	—	F	
	Spio martinensis	Hannerz, 1956	Sy	P	F	Adults differ in size; larvae in pigment and morphology. Probably 2 species.
		*Mesnil & Caullery, 1917	S	N, P	F	
	Spio setosa	*Simon, 1968	S	B, P	L	Young cultured from plankton. Adults not associated. Larvae differ in setae, pigment, etc.
		Hannerz, 1956	—	—	F	
		Blake & Kudenov, 1981	S	N, P	—	
	Boccardia acus	*Read, 1975	—	N, P	F	Morphological differences between 2 populations.
	Boccardia androgyna	*Read, 1975	—	N, P	F	Percent nurse eggs varies; may be none. Constant within an egg string.
	Boccardia chilensis	*Read, 1975	—	N, P	F	Two types cited but no data.
		*Blake & Kudenov, 1981	G	N, P	—	
	Boccardia otakouica	*Read, 1975	—	N, P	F	No larvae found in plankton. Percent nurse eggs varies.
	Boccardia proboscidea	*Hartman, 1941	Sy	N, P	L	Some morphological differences.
		*Blake & Kudenov, 1981	Cp	N, P	L	

Table II (Continued)

Family	Genus & species	Citation	Type	Larval forms	Evidence	Comments; status
Spionidae (cont.)	*Pygospio elegans*	*Hannerz, 1956	S	N, P	FA	Spring, summer difference in length of pelagic stage reported.
		*Rasmussen, 1956	Sy	N, P	F	
		Thorson, 1946	Sy, G	N, P	F	
		Söderström, 1920	S	N, P	F	
	***Streblospio benedicti*	*Levin, 1984	G, S	B, P	LB	Breeding of 1 generation.
Cirratulidae	*Cirratulus cirratus*	*Fauvel, 1916	G	Br	FA, I	Falklands vs. North Seas.
		Cunningham & Ramage, 1888	G	B	L, I	
	Dodecaceria concharum	**Thorson, 1950	Sy, G	Br, B	FA, I	Total of 5 types of reproduction, 3 of them sexual. Martin (1933) concluded species complex.
		Dehorne, 1927	G	Br, B	FA	
		*Mesnil & Caullery, 1898a, b	Sy	Br, E-P	FA	
	***Cirriformia tentaculata*	*George, 1963, 1967	G	B, P	L	Adults differ in size (George, 1963). Single female produced both types (George, 1967).
			Sy	B, P	LB	
Terebellidae	*Eupolymnia nebulosa*	Gremare, 1986	G	B, P	FA	No discussion of taxonomy or morphology.
Capitellidae	*Capitella capitata*	*Rasmussen, 1956	Sy	N, P	F, L	Grassle & Grassle (1976) demonstrated a species complex.
Arenicolidae	*Arenicola claparedei*	*Okuda, 1938	G	B, P	F, I	Japan vs. Puget Sound. Found to be 2 species (Healy & Wells, 1959).
		Guberlet, 1934	—	—	F	
Serpulidae	*Pomatoceros triqueter*	*Gravier, 1923 v.	G	P	F, I	Short pelagic stage.
		Drasche, 1884	—	Br	F	

Nassariidae, Turridae, and Conidae. All major groups of opisthobranchs also have such genera (Thompson, 1976), as do many bivalves (the Teredinidae are just one example).

Several forms of reproduction occur in many genera of polychaetes, but especially those of the Capitellidae, Spionidae, Nereidae, and Syllidae (Mileikovsky, 1971; Wilson, 1928). Two kinds of development occur in the archiannelid genus *Protodrilus* (Schroeder and Hermans, 1975). Sipunculids of the genus *Golfingia* can undergo direct development or can release pelagic trochophores (Rice, 1967). In enteropneusts, direct and indirect development correlate with taxonomic grouping at the family level (Hadfield, 1975). Both sponges and tunicates can be oviparous or viviparous at various taxonomic levels (Fell, 1974; Berrill, 1975). Some species of ctenophores have brood chambers (Pianka, 1974). Nemertinea have species with pelagic and species with non-pelagic larvae (Riser, 1974); there are also several kinds of larvae in the polyclad flatworms (Henley, 1974).

Many crustacean genera have species with differing modes of development. For example, one abyssal family of decapod crustaceans contains a few species with pelagic young while most are benthic; the same is true in abyssal echinoderms (ophiuroids). As an example from decapods, both brooding and pelagic development are known in the genus *Sclerocrangon* (Makarov, 1918 in Mileikovsky, 1971). Many echinoderm genera including *Asterias*, *Asterina*, *Heliocidaris*, and *Echinocyamus*, as well as several ophiuroids, have more than one mode of development, usually viviparity and ovoviviparity (Mortensen, 1921).

Discussion

Most reports of poecilogony have been made by well-known and respected marine biologists, yet few reports have come from workers who are or were primarily systematists. The available data on poecilogony are scant. Nonetheless, examples have been quoted repeatedly in the literature, obscuring the speculative nature of the original reports. For example, Houbrick (1973) has been quoted to say that poecilogony occurs in *Cerithium*, but actually he did not find any evidence for it. He only suggested that researchers look for examples. The impression has been given that there are cases in which environmentally induced variation of the amount of yolk or nurse-egg material leads to different developmental stages at hatching (Clark *et al.*, 1979), yet we found no example in the literature that was backed by data. Clark and Jensen (1981) stated "*If* hatching is controlled by exhaustion of nutrient reserves, then change of albumen content *could* provide a simple mechanism for extending or abbreviating intracapsular development in response to environmental change . . . even single egg masses *may* yield different larval types" [italics ours]. In support of the hypothetical statement are three references; one of these in turn cites a "personal communication," and the other two we judge irrelevant to the subjects of poecilogony and control of hatching. Jensen and Clark (1983) elsewhere use the pattern of development of the ascoglossan *Oxynoe antillarum* to distinguish it from *O azuropunctata*.

Whether a particular species has two different types of larvae produced by the same female (at different times or from the same egg capsule) is the critical question. Thorson (1946) concludes that such cases exist, yet supports his statement with text describing *allopatric* populations. Hadfield (1972) states that in the vermetids ". . . some larvae from a single capsule will hatch with, others without, a velum. . . ," yet the only published account we have read is of veligers found in plankton samples (Hadfield *et al.*, 1972). In none of the cases in Tables I and II has a single female been proven to produce two kinds of hatching offspring, except when metamorphosis occurs within a day of hatching.

Production of viable offspring by parents with different types of reproduction (that is, individuals sharing a common gene pool) is sufficient to demonstrate poecilogony. Only two breeding experiments in polychaetes and two in gastropods have been attempted, and all are incomplete in that they lack published genetic data in the form of allozyme or other studies. It is particularly important that genetic data be included to test claims of geographic or seasonal poecilogony. Care must be taken in interpreting breeding data for opisthobranchs and other invertebrates that can possibly self-fertilize, and with all invertebrates that store sperm.

Circular reasoning is easy when analyzing cases of poecilogony. If one is inclined to believe that poecilogony occurs, an example of two types of reproduction in morphologically similar adults is taken on face value as poecilogony without rigorous systematic analysis. On the other hand, if one is inclined to doubt poecilogony, the same example is taken to demonstrate the existence of two species. We suggest that each putative case of poecilogony should be a flag to alert researchers to the need for careful systematic study, including anatomical and molecular genetical analyses. Studies on interbreeding are particularly important.

Taxa that retain the early larval stages, *e.g.*, the velum in mollusks, within an egg capsule are more likely to reverse the evolution of capsular development (Strathmann, 1978, 1979, 1985). A change between planktotrophy and lecithotrophy in polychaetes requires changes in swimming setae and other features. Thorson (1950) correlated the proposed ecological plasticity of mode of reproduction in polychaetes with their cosmopolitan distributions; however, this result might well have been due to errors in polychaete systematics. Some early polychaete workers such as Fauvel had a rather broader species concept than we do today. In polychaetes, taxonomists have traditionally ignored reproductive and juvenile characteristics as taxonomic characters. Smith (1958) pointed out that to do so is to ignore potential reproductive isolation. He also emphasized that *mature* adult morphology should be used to compare species; this includes reproductive specializations such as the characters of heteronereids. *Nereis japonica* and *N. limnicola* differ morphologically at sexual maturity, but not in the non-reproductive state (Smith, 1958). Careful systematic work in gastropod taxa such as the Littorinidae, Rissoidae, and the Aeolidiidae have greatly reduced the number of potential cases of poecilogony (Heller, 1975; Rivest, 1978).

It is interesting that size of adults is bimodal in many mollusks and polychaetes thought to have poecilogony (*Littorina saxatilis, Tenellia pallida, Spio martinensis, Cirriformia tentaculata*). In every case, the smaller adults produce non-pelagic young; the larger adults produce pelagic young. Rather than invoking a shift in egg type with age or size, one can as easily hypothesize that different species are involved. Similarly, seasonal shifts in mode of reproduction might be explained by poecilogony (Mileikovsky, 1971 for *Littorina angulifera*), but the staggering of egg production by sympatric congeners is a common phenomenon in marine invertebrates and must be considered to be a viable alternative hypothesis.

Several theoretical papers have dealt with poecilogony, or with the problem of evolution of modes of larval development. Some (Vance, 1973a, b; Christiansen and Fenchel, 1979; Grant, 1983) conclude that there are dichotomous adaptive egg sizes, but these authors do not provide a model for the evolution of poecilogony within a species. Kishi's (1979) model of disruptive selection on offspring size (of fish) could be used to explain poecilogony as an intermediate stage in speciation for those taxa in which neither larval form possessed specialized structures. Many papers (*e.g.*, Underwood, 1974) do not clearly distinguish between intra- and interspecific variability in reproductive characters. Knowledge of the true extent of intraspecific variation in existing populations could illuminate proper theoretical approaches. Caswell (1981) was misled by erroneous examples of poecilogony given by Spight (1975) and Gallardo (1977) into overemphasizing the switching of a species from one form of development to another.

The theoretical advantages of poecilogony are often cited, *e.g.*, "Pelagic larvae are dispersed by tides and currents, exposing individuals to new habitats. Once established, non-pelagic larvae produced by these newly-settled forms may rapidly establish large populations themselves . . ." (Blake and Kudenov, 1981, p. 181). The assumption is that a single genetic lineage can switch developmental mode to its best ecological advantage. In no case has such switching been demonstrated in the field or laboratory.

The rarity of examples of possible poecilogony in marine invertebrates is perhaps surprising, when we consider the theoretical framework for the concept. First of all, there *is* great intraspecific variability in other reproductive characters, such as brood size, fecundity, age at maturity, and sometimes, egg size (Brown, 1983). But these are all quantitative differences, while poecilogony is, by definition, a qualitative difference involving ecological as well as morphological differences. Secondly, the number of cases of congeneric reproductive differences shows the relative ease of evolutionary shift of reproductive modes. But reproductive change of the type required by poecilogony could lead quickly to reproductive isolation, if offspring of an intermediate type were dysfunctional.

Valentine and Jablonski (1982) theorized that a shifting proportion of individuals within a population with genetically determined longer or shorter larval lives was responsible for the evolution of mode of reproduction based on local selection. The mechanisms of nurse eggs and extracellular yolk would seem to provide a way to slide gradually from one reproductive strategy to another without dysfunction, yet we cannot demonstrate from available data that this is happening in any single population. Tests of the theory could be made using members of the prosobranch gastropod families Calyptraeidae with nurse eggs, the opisthobranch ascoglossans, the Vermetidae, and the spionid polychaetes.

Conclusion

Our literature review leads us to conclude that poecilogony is at best poorly documented as a phenomenon in marine invertebrate reproduction. The pattern of development is indeed a generally reliable species character in invertebrate systematics, and differences often signify unrecognized species. While shifts in egg nutrition by varying extracellular yolk or nurse-eggs is an attractive way to explain evolution of reproductive modes, we have no examples of facultative shifts occurring in modern populations, nor do we find clear examples of polymorphism in type of reproduction within a single population of marine invertebrates. Therefore it appears that shifts in mode of reproduction usually occur rapidly and completely within populations, leading to reproductive isolation and hence speciation.

One example of poecilogony in the literature appears to represent incomplete reproductive isolation in allopatric populations (*Elysia chlorotica*). Poecilogony in *Streblospio benedicti* could represent a recent contact between formerly allopatric, speciating populations. More breeding experiments are needed for *Tenellia pallida* and *Cirriformia tentaculata* to verify cross-fertilization in sympatric populations, but poecilogony is not ruled out. The fate of egg types in the natural environment must be known for *Boccardia proboscidea* to know if it is an example of adelphophagy, poecilogony, or both. One other possible type of poecilogony has been found: some species produce very short-term lecithotrophic veligers that metamorphose a few hours after hatching; sometimes some individuals hatch while metamorphosing (*e.g.*, *Murex incarnatus*). All other cases of poecilogony in the literature have been disproven, or are not supported by sufficient data to judge them.

To support the hypothesis of poecilogony, at least one of three kinds of data is needed:

1) Genetic (allozyme) data for sympatric individuals, to see if interbreeding occurs between individuals with different larval types or if the individuals are fixed for alternate alleles and hence are *not* interbreeding.
2) A single individual observed in the laboratory to produce two types of larvae, *i.e.*, under varying environmental or nutritional conditions.
3) Cross-breeding data for normally allopatric populations, combined with allozyme or other molecular genetic studies, to show that (a) cross-fertilization occurred, (b) viable offspring were produced, (c) the offspring in turn are capable of breeding and reproducing with each other and the parental types, and (d) the cross-breeding event could plausibly occur in nature as well as in the laboratory. The heritability and genetics of mode of larval development need to be established.

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Entrainment of Tidal and Semilunar Rhythms by Artificial Moonlight Cycles

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Abstract. A marked feature of the larval release activity of the terrestrial crab *Sesarma haematocheir* is its synchronization with the time of high water. This activity occurs only at night, so that the pattern of the tidal rhythm recurs at semi-monthly intervals. When adult specimens from Seto (Okayama Prefecture) and Shima (Mie Prefecture) populations were brought from the field into 24-h light-dark conditions in spring, the larval release occurred at night, but the overall activity pattern gave no indication of a tidal component. On the contrary, under simulated moonlight cycles the timing of release was strongly coordinated and exhibited a well-defined tidal component arranged at semi-monthly intervals. The phase difference between the evoked tidal rhythms of the two populations of 4–5 h was about equal to the phase difference of the tidal cycles in their natural habitats. Synchronization of larval release with the artificial moonlight cycle required more than 40 days of exposure. In addition to entraining the tidal rhythm, artificial moonlight induced a semilunar rhythm in both populations. Entrainment could be achieved with exposure to moonlight for just a few days around the time of the full moon. In this paper, underlying mechanisms of the *Sesarma* larval release rhythm, which involves both tidal and semilunar components, are explained in terms of circadian oscillatory systems.

Introduction

From the viewpoint of ecological adaptation of biological timing systems, circa-tidal rhythms are known to respond to environmental stimuli correlated with on-shore tides. Enright (1965) demonstrated that cycles of water turbulence, which simulate waves washing over the beach, can effectively entrain the circa-tidal activity rhythm of the isopod *Excirolana.* Other experiments indicated that cycles or changes of hydrostatic pressure cause behavioral responses in the amphipods *Synchelidium* (Enright, 1962) and *Corophium* (Morgan, 1965), and the pychnogonid *Nymphon* (Morgan *et al.*, 1964). However, these experiments did not show that such stimuli can entrain endogenous rhythmicity of the animals. Environmental variables coinciding with tidal cycles such as temperature might also affect synchronization processes.

The larval release activity of the terrestrial crab *Sesarma haematocheir* coincides with the times of high water at night, showing a unimodal tidal rhythm. A phase jump is involved in the timing process around the first and last quarters of the moon, so that the pattern of the tidal rhythm appears at 15-day intervals (Saigusa, 1982, 1985). When ovigerous females from the Seto population (Okayama Prefecture) are introduced by continuously dark conditions in the laboratory, they show a free-running rhythm whose phase at least initially is synchronized with times of nocturnal high water on the shore. The fact that the phase jump to the conjugated high water is reproducible under constant conditions suggests that its timing is also controlled endogenously. Artificial 24-h light-dark cycles cause a phase-shift of the circa-tidal rhythm. A striking aspect of the activity record is that the degree of the observed phase-shift closely corresponds to the time difference between lights-off and sunset. This indicates that 'dusk' is the critical signal for phase-setting in the circa-tidal timing systems of *Sesarma* (Saigusa, 1986).

A 24-h light-dark cycle thus functions as a zeitgeber (environmental cue), of *Sesarma* circa-tidal rhythm. However, there should be environmental cues other than

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the day-night cycle so that the daily timing of larval release may be synchronized with nocturnal high water. Two possible synchronizing agents were considered (Saigusa, 1985): (1) periodic changes in vibration or sound of surf washing on the beach, although Enright (1972) denied such a possibility; and (2) moonlight cycle, which is the zeitgeber for a semilunar rhythm. Previous experiments (Saigusa, 1986) were unable to demonstrate definite synchronization of *Sesarma*'s rhythm by artificial tidal cycles. Thus the next step was to determine whether this rhythm can be entrained by artificial moonlight.

This study attempted to establish the manner in which the endogenous tidal rhythm of *Sesarma* is synchronized by environmental cycles. For the 'control' experiment, populations of crabs were kept from spring in a 24-h light-dark cycle in the laboratory. Females that incubate eggs under these conditions are expected to lose synchrony with each other and show no clear tidal component in the larval release activity. In another experiment, crabs were maintained for several months under artificial moonlight cycles. If moonlight can synchronize *Sesarma*'s rhythm, the timing of release would be strongly coordinated to show a definite tidal component, and the requisite phase relations between the observed tidal rhythm and the artificial moonlight cycle would be the same as those between the tidal rhythm and the moonlight cycle in the field. Or if moonlight is an entraining agent of the tidal rhythm and the synchronization is possible up to the second or third release of larvae, then it may be possible to entrain a free-running rhythm of freshly collected populations. Furthermore, it may be possible to determine what component involved in moonlight cycles is actually significant for entrainment. Thus entrainment of a tidal rhythm of *Sesarma* by artificial moonlight cycles is the main focus of this article.

Saigusa (1980) showed that artificial moonlight cycle entrains a semilunar rhythm of larval release, *i.e.*, a remarkable semi-monthly variation in the number of females releasing larvae per night. However, this study was made using the Shima population, an inhabitant of the seacoast of the Pacific Ocean. It is unknown whether simulated moonlight cycles can similarly induce a semilunar rhythm in the Seto population which inhabits the seacoast of the Inland Sea. If artificial moonlight cycles evoke not only the tidal rhythm but the semilunar rhythm in both populations, then a question arises as to what relationships exist between the tidal and semilunar timing systems.

Field observations (Saigusa, 1982, 1985) demonstrated that the timing of larval release is synchronized with local tidal cycles. The habitat of the Seto population is situated around the central part of the Inland Sea, so that tidal cycles are delayed 4.5–5 h from those at Shima. The question also arises as to how moonlight cycles synchronize *Sesarma*'s rhythm and local tidal cycles.

Materials and Methods

Adult male and female crabs (*Sesarma haematocheir*) were collected from the field at Kasaoka, Okayama Prefecture, and Gokasho, Mie Prefecture. Collection sites are described elsewhere (Saigusa, 1980, 1982). In autumn crabs migrate to the hill near the riverside where larval release is performed, and hibernate in burrows dug on the ground or in narrow spaces among rocks or heaps of stones until the end of April. Crabs were dug out of the ground until April and captured in the thickets thereafter. Crabs were then transferred to a light and temperature controlled laboratory (luminous intensity at about 700 or 1200 lux on the floor in the light period of 24-h LD cycles). Crabs were placed in an aquarium equipped with a shallow pool on one side (to facilitate ecdysis) and hiding places made of boards on the other. Crabs were fed daily. Temperature was maintained at 23 ± 1.5°C for most experiments.

Once copulation is finished, ovulation occurs and fertilized eggs are attached to ovigerous hairs in the folded abdomen; this is termed 'onset of incubation' or 'egg-production.' The female then carries a clutch while eggs undergo embryonic development. Each female was examined daily for the onset of incubation, and ovigerous females were separated in small plastic containers (70 cm long, 40 cm wide, and 25 cm high) containing a small amount of very diluted seawater (see Fig. 1 in Saigusa, 1980). The color of the eggs was examined every few days. Females with lustrous, brownish green eggs (signaling the onset of larval hatching) were transferred to another container. Those animals whose eggs were estimated to hatch within a few days were individually placed in the recording apparatus to monitor the time of larval release.

The larval release recording system consisted of a sensor unit (infrared source-receiver) placed within the experimental chamber, and a photoelectric-switch-amplifier unit placed outside the chamber. The latter unit detects and responds to a decrease in the transmitted light beam following the release of zoea-larvae. In the present experiments OPE-S100 or E3S models (Omron Co. Ltd., Japan) were used as the sensor unit. The output of the photoelectric switches was monitored by an event recorder (Saigusa, 1986). Crabs were confined within the chamber throughout the experiment.

An incubating crab was placed in the recording apparatus until larval release occurred. The glass beaker was replaced and a new ovigerous female was transferred from the container to the recording apparatus after larval release. This procedure was performed during the light period of 24-h LD cycles. The females who had completed larval release were placed in another container where they incubated the next clutch without males. Subsequent procedures were the same as those for the first incubation. Experiments were performed using four chambers fitted with 6–15 recording instruments.

To test whether the daily timing of larval release by females can be affected by moonlight, the chamber was illuminated with dim light produced by a midget lamp—the same used for entrainment of a semilunar rhythm in the Shima population (Saigusa, 1980). The intensity of this artificial moonlight remained constant at 0.1–0.15 lux at the floor and thus did not mimic the phases of the moon.

The trend of activity pattern produced by the treatment of the artificial moonlight was estimated by calculating least-squares regression lines through sequential activity records. The slope of each regression line (y = ax + b) is given by the following equation:

$$a = \frac{\Sigma(x_i - \bar{x})(y_i - \bar{y})}{\Sigma(x_i - \bar{x})^2}$$

where: x_i and y_i indicate a coordinates used to express each point of the larval release activity (the origin is taken on an intersection of the date-axis and the time-axis); $\bar{x}$ and $\bar{y}$ show mean values of x_i and y_i, respectively. The deviation of each point of the activity from the regression line was estimated along the abscissa where the point is plotted, and the variance (V) of those estimates was calculated for every regression line. In this paper, this value (a unit: h) is used as an index of the degree of synchronization among individuals.

Results

For the 'control' experiment, male and female crabs were collected at Kasaoka on 6 May 1984 and confined in the experimental chamber. They were kept under an artificial light regime (LD 14:10) which does not include moonlight. Under this condition the first incubation occurred from May to July. Each female incubated eggs for one month and then released them as zoea-larvae. Most females subsequently carried a second clutch and released larvae after the same period. Larval release occurred at night (Fig. 1)—especially in the latter half of the night. The release was not synchronized and the overall activity pattern gave no clear indication of a tidal component.

To evaluate the effect of artificial moonlight cycles on the timing of larval release activity, crabs (60 males; 100 females) were collected at Kasaoka on 24 April 1983 and transferred to the laboratory. They were kept under a light regime which included a 24.8-h artificial moonlight cycle at night. The initial photoperiod applied to the animals was LD 12:12. Under this condition 30 females started their first incubation from the 22nd to the 37th day and released larvae between the 53rd and the 66th day (see Fig. 5B). As indicated in Figure 2A, the simulated moonlight cycle brought marked changes in the daily timing of the activity: a negative slope is clearly seen from the nocturnal activity pattern.

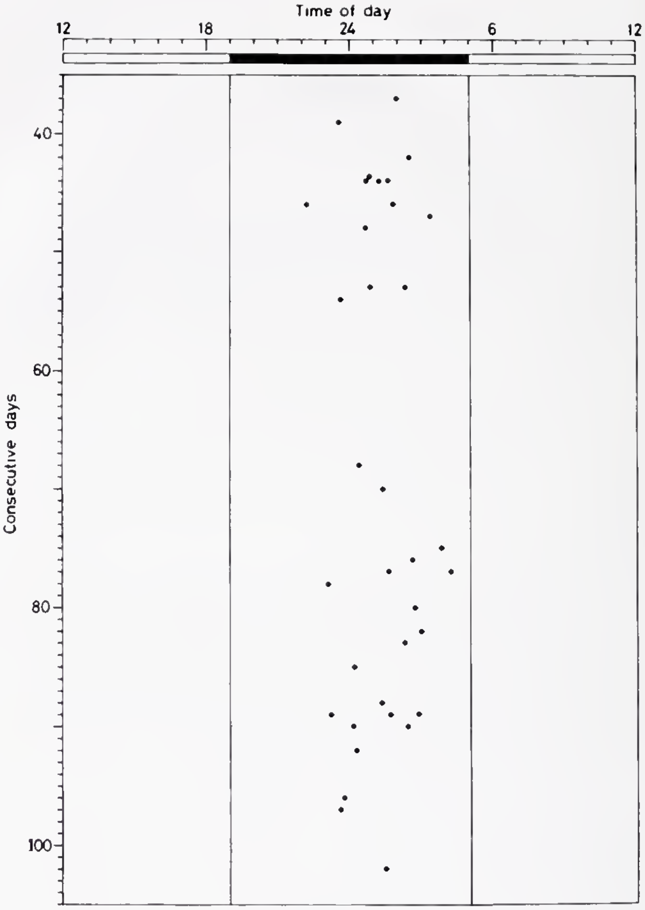


Figure 1. Daily timing of larval release by the Seto population monitored under the condition of a 24-h LD alone. A black dot indicates the time of day of larval release by a female. Vertical axis: number of days after the population was introduced to the laboratory.

In this experiment no incubation occurred after the 38th day (1 June). Since it was evident that the short-day condition inhibited egg-production, the day-length was changed to LD 15:9 from the 67th day (30 June). This treatment induced ovulation after two weeks. Such an inhibitory action by the photoperiod also occurred to the animals shown in Figure 2A: these females did not incubate the second clutch immediately after the release of larvae, but required at least one month of exposure to the long-day conditions.

The artificial moonlight program adopted in the short-day conditions (Fig. 2A) was composed of an idealized period of 24.8 h. For long-day conditions, however, the

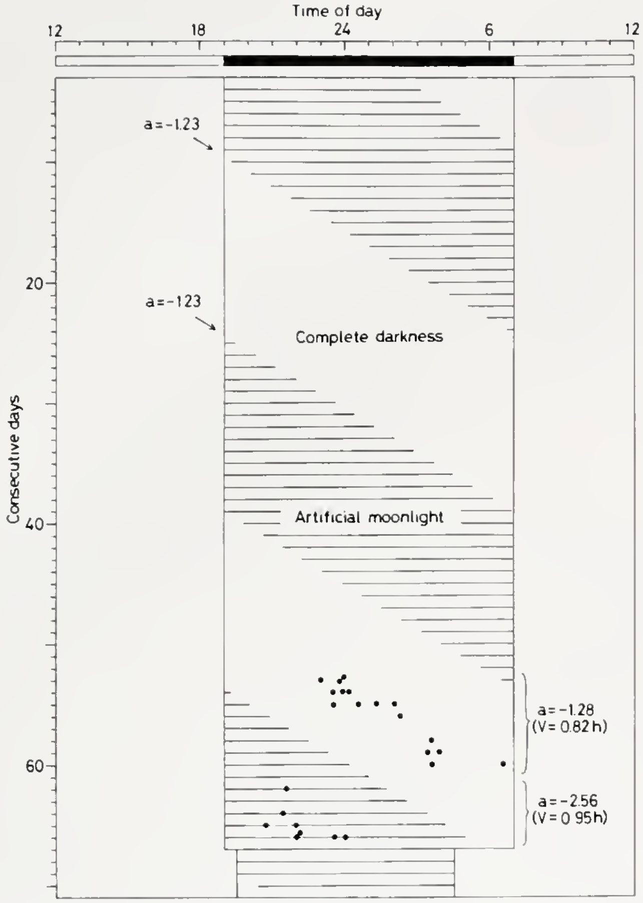


Figure 2(A). The larval release activity by the Seto population subjected to an artificial moonlight cycle of 24.8 h. Horizontal bars show the times when the experimental chamber was illuminated by the artificial moonlight. 'On' and 'off' times of this moonlight were manually set every day using a timer. *a:* The slope of retardation of 'on' or 'off' of the artificial moonlight (left side), and the slope of each tidal component (right side). *V:* Variance of the spawning activity along the regression line.

period of the simulated moonlight cycle had to be modified so that it could recur at 15-day intervals. In this experiment, such a modification was based on data from the Annual Table on Scientific Affairs (Japanese name: Rika Nenpyo, ed. by Tokyo Astronomical Observatory): times of 'rise' and 'fall' of the moon were replaced by the times of 'on' and 'off' of the artificial moonlight, respectively. This is the same kind of zeitgeber program as used previously for entrainment of a semilunar rhythm (Saigusa, 1980). Of the data described in this table, those from June–September were continued from the initial program.

Figure 2B summarizes the daily timing of larval release activity recorded under the new light regime which included a 24.5-h artificial moonlight at night. At first glance the activity corresponds to the phase of the moonlight cycle, showing a pattern with a negative incline at semi-monthly intervals. In this population, the slope of each sequential activity pattern was close to that of the artificial moonlight cycles. Thus the fact that the activity of a group of crabs was transformed from apparently arrhythmic (Fig. 1) to clearly coordinated (Figs. 2A, B) demonstrates that the moonlight cycle is adequate to be the zeitgeber of the circa-tidal rhythm of *Sesarma.*

Field studies (Saigusa, 1982) established that the tidal rhythm of the Seto population causes a phase jump of 7–

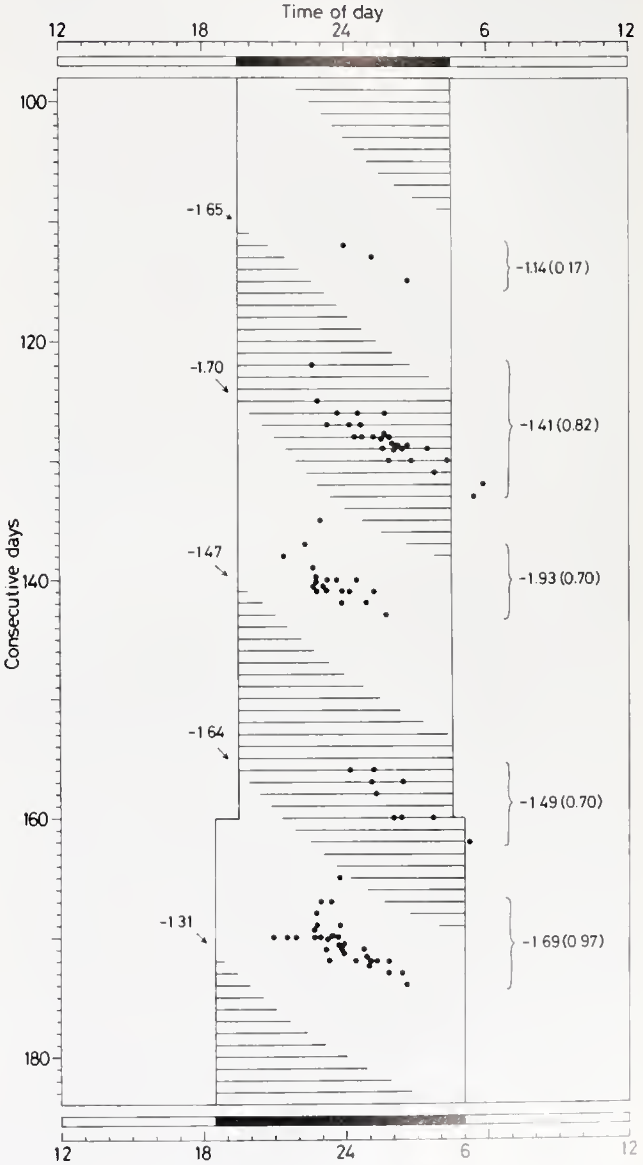


Figure 2(B). Daily timing of larval release by the Seto females recorded under an artificial moonlight cycle of about 24.5 h. Vertical axis: number of days after the population was brought from the field (the data continued from Fig. 2A). Slope of each tidal component and variance of the activity are shown on the right side of the corresponding data (the data on days 135 and 165 are not included in calculation of regression line). Temperature was constant in the region of 21 ± 1.5°C until day 65, and about 23 ± 1.5°C thereafter.

8 h around the first and last quarters of the moon. The activity appears immediately after dusk for 3–4 days before the afternoon high tide advances to the night. The long-duration records in the laboratory, however, showed no clear indication of the timing synchronized with the onset of darkness. The records of days 165–168 illustrated in Figure 2B, for instance, show that the activity occurred more than 4 h after lights-off. Furthermore, as seen in the records of days 133–134 and day 162, the activity occurred beyond the dark period. These phenomena, which have not been observed in the field, happened often during the experiments using the Seto population.

Experiments to examine the effect of artificial moonlight cycles on the daily timing of the activity were also conducted using the Shima population. For this purpose male and female crabs were captured on 7–8 May 1986 from the field and transferred to the laboratory. They were divided into two groups: one was kept under the artificial light regime without moonlight (LD 15:9), and another was exposed to a 24.5-h artificial moonlight at night (LD 15:9). The group kept for a long time under a 24-h LD condition alone is expected to show no clear tidal component in the activity pattern. On the other hand, if moonlight is an essential stimulus synchronizing *Sesarma*'s rhythm, the group exposed to artificial moonlight would exhibit a distinct tidal component at night.

Without moonlight, Shima females released larvae at night; most release occurred during the first half of the night (Fig. 3). The activity pattern did not show a detectable tidal component throughout the period. However, another group from the Shima population synchronized its release to the artificial moonlight cycle (Fig. 4), yielding a distinct tidal component at semi-monthly periods. A striking feature on comparing the data illustrated in Figure 4 with those of Figures 2A and B is that the evoked tidal rhythms in the two populations had different phase relations to the artificial moonlight cycles; the phase difference was about 4–5 h. The fact that the slopes of the tidal component in the Shima population were larger than those of the Seto population would mean that the period of the Shima population tidal rhythm is closer to that of a so-called daily rhythm.

Figure 5A summarizes the variation of the number of Shima females releasing larvae per night. The 'control' experiment (upper panel) group did not show a clear rhythmicity either in the onset of incubation or in the timing of larval release. On the contrary, the group treated with artificial moonlight (lower panel) exhibited a well-defined semi-monthly variation in the larval release activity, and the fluctuation corresponded to the varying phase relations between the 24-h LD and artificial moonlight cycles. For this group the number of females that started incubation also fluctuated at semi-monthly intervals. The semilunar rhythm of ovulation is certainly weaker than that of the larval release. Moreover, when the onset of incubation was delayed (an arrow indicated in Fig. 5A), some females were included in the group animals that constituted the next peak of the larval release. Nevertheless, the trend in Figure 5A suggests that the semilunar rhythm of larval release is ba-

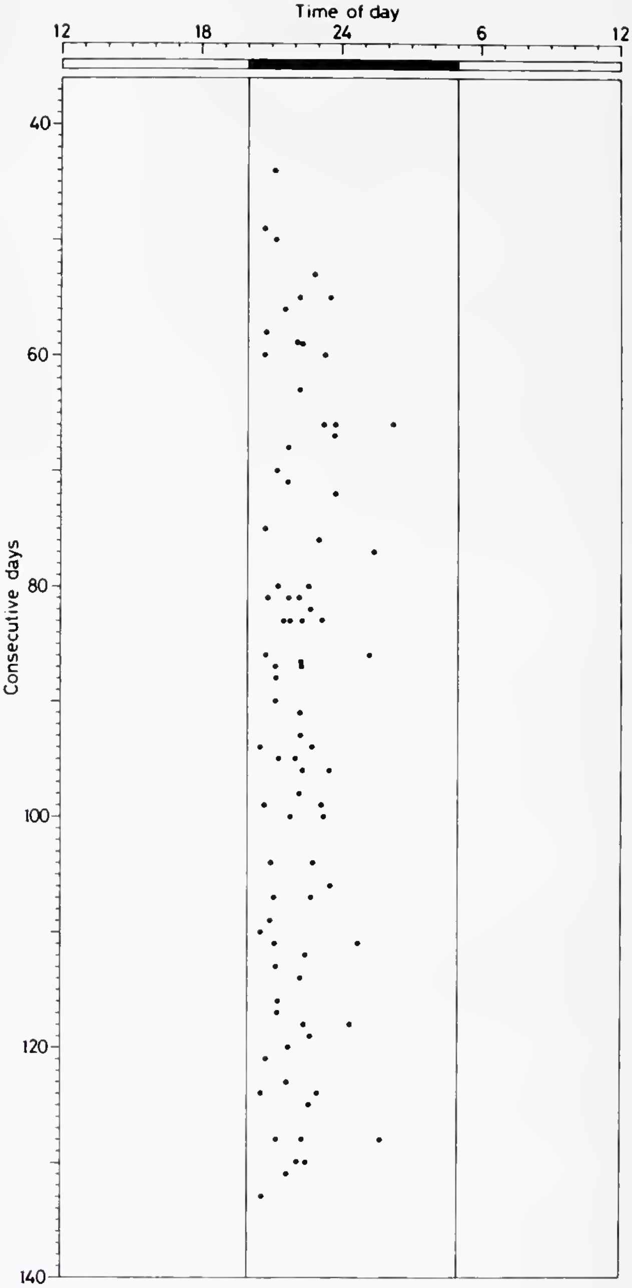


Figure 3. The larval release activity of the Shima population monitored under the condition of a 24-h LD cycle containing no moonlight. The data show two spawnings by most females and a third spawning by a portion of them.

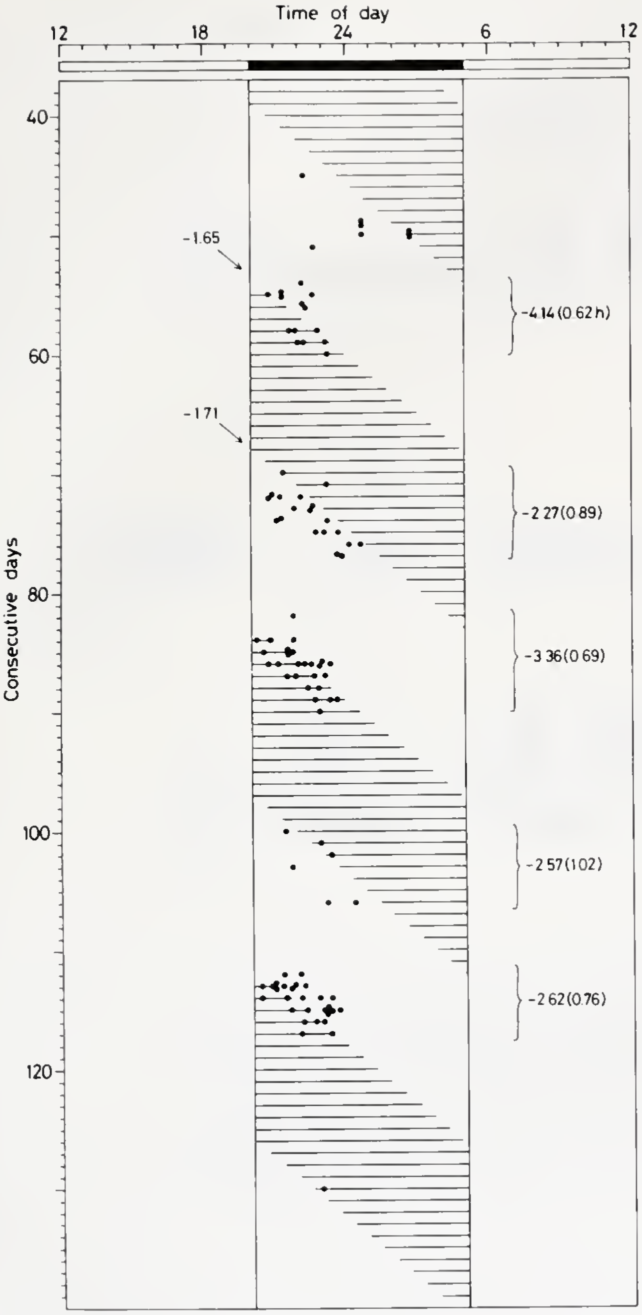


Figure 4. The larval release activity of the Shima population subjected to an artificial moonlight cycle of about 24.5 h. Horizontal bars indicate the artificial moonlight. Slope of the retardation of this moonlight is shown by arrows on the left side of the data. Slope and variance in each tidal component are indicated on the right side of the corresponding data. Vertical axis: number of days after 8 May.

sically due to the occurrence of the semilunar rhythm of ovulation.

A similar fluctuation was also obtained from the data of the Seto population treated with artificial moonlight (Fig. 5B). In this experiment a short-day condition inhibited egg-production so that no female released larvae between the 68th and the 111th day. In a long-day condition three distinct peaks (A, B, and C) and a small peak (D) appeared in the larval release activity. Peaks A and B consist of the spawning by the females which first began incubation during the long-day condition and the second spawning by females that hatched out the first brood during the short-day condition, respectively. Most females in group A were added to group B at the onset of the second or third incubation, so that the peak D became vague. Each peak of the larval release activity in this population is not so clearly separated as that of the Shima population semilunar rhythm (Fig. 5A). This suggests that the semilunar timing of the Seto population is somewhat weak compared with that of the Shima population.

Previous experiments (Saigusa, 1986) demonstrated that the timing of larval release in *Sesarma* is controlled endogenously, thus the synchronized release in Figures 2A, B, and 4 is not a direct response of the animals to the moonlight stimuli. But the question remains: what component of the moonlight cycles synchronizes *Sesarma*'s tidal rhythm? Since the moonlight cycles in Figures 2 and 4 are unnatural due to a lack of cyclic variation in light intensity, it is possible that the tidal rhythm is synchronized by (1) moonlight illumination for some days around the full moon (*ca.* 30-day period), or (2) by retardation of the times of rise and/or fall of the moon (*ca.* 24.5-h to 24.8-h period).

To answer this question, the animals were exposed to two kinds of simulated moonlight cycles. At first, effects of the moonlight at the time of the full moon were examined. Crabs were collected at Kasaoka on 24 April 1986 and exposed to artificial moonlight throughout the night (9 h) for one week every 30 days. If the retardation of the times of rise and fall of the moon is important for entrainment, then the larval release activity should show no definite tidal component under such a condition. However, the results (Fig. 6A) indicated a tidal component at semi-monthly intervals. As seen from values of the variance, individual animals are not so strongly synchronized as those monitored under 24.5-h moonlight cycle (Fig. 2B). This indicates that such a moonlight cycle (Fig. 6A) weakly entrains *Sesarma*'s rhythm.

In the next two experiments the same kind of zeitgeber program was adopted for both Shima and Seto populations collected on 11–12 May and 14 May 1987, respectively. As indicated in Figures 6B and C, each chamber was illuminated by dim light through the night (9 h) for four days each month. Unlike the results shown in Figure 6A, the tidal component was not detectable from either of these experiments. In another experiment the Shima population (collected 11–12 May 1987) was exposed to a moonlight cycle simulating the time of the new moon. This experiment also failed to demonstrate a well-de-

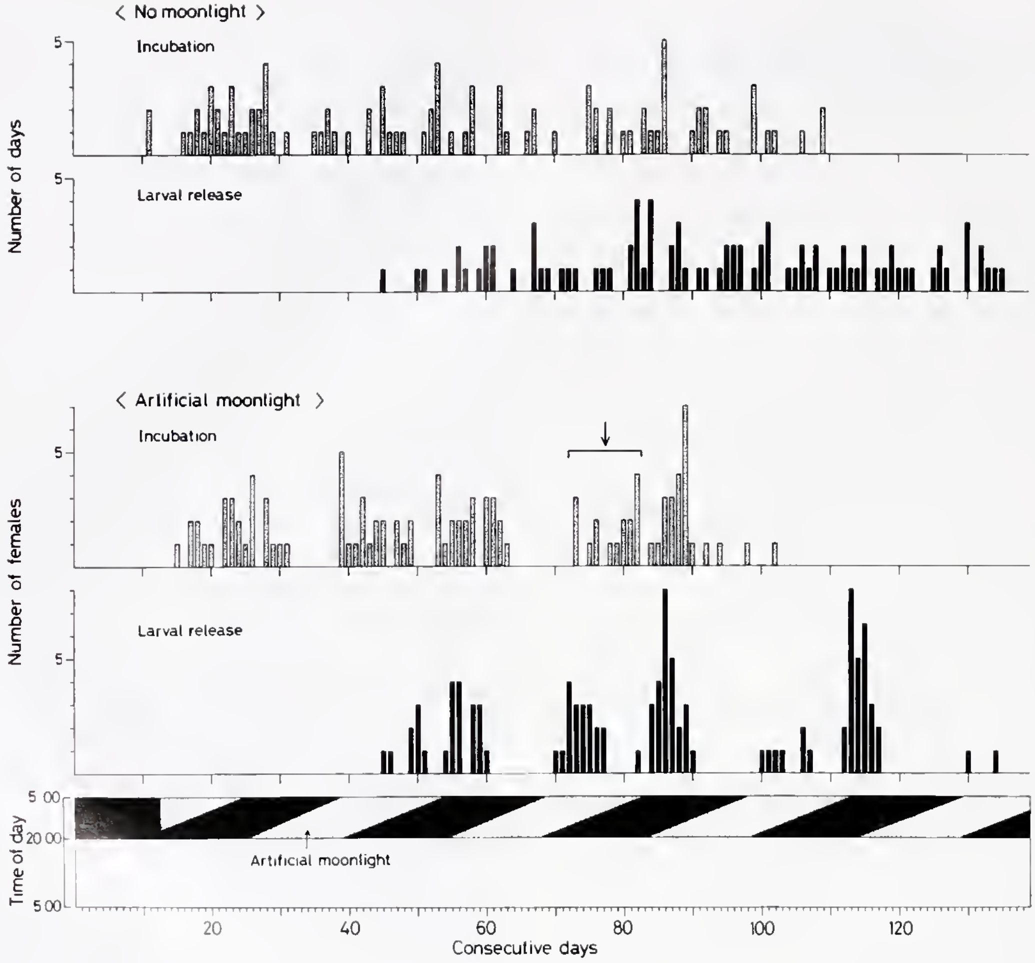


Figure 5(A). Plots of the number of females starting incubation per day and the number of females releasing larvae per night (Shima population). Upper panel: experiments without moonlight. Lower panel: experiments with a 24.5-h artificial moonlight cycle. Moonlight program is shown at the bottom of the figure. The data of larval release are based on those of Figures 3 and 4, respectively. The number of females does not precisely agree with the number of black circles in corresponding dates of those figures, because there were some females whose larval release was not monitored. All females were marked in these experiments. Periods of incubation in each female (upper panel) were 30.4 days for the first brood, 27.8 days for the second brood, and 26.8 days for the third brood, respectively, in the mean. Incubating periods were similar in the group exposed to moonlight (lower panel): 30.9 days, 28.7 days, and 27.0 days, respectively.

fined tidal component (Fig. 6D). These data probably suggest that moonlight cycles that simulate either the full moon or the new moon have a lesser effect on entrainment than the moonlight cycle shown in Figures 2 and 4.

The reason a tidal component is seen in one case (Fig. 6A) and not the other (Fig. 6B) for same population is because of the difference in the time of collection. While the former experiment (Fig. 6A) began on 24 April, the latter (Fig. 6B) commenced on 14 May. (In both experiments artificial moonlight was administered around the time of the half moon. In the former experiment the first moonlight cycle was supplied before the full moon in May, *i.e.*, around the first quarter; in the latter experiment it was given around the last quarter in May.) Crabs would have been more strongly synchronized with natural moonlight cycles during the three weeks. This would have delayed entrainment in the latter experiment, which made it difficult to evoke a tidal rhythm during the three times of spawning.

Figure 7 summarizes the fluctuations of the number of females that released larvae under these zeitgeber programs. A weak semilunar rhythm is distinguishable from the data of the Seto population collected in April (Fig. 7A), but no clear rhythmicity appears in the other data (Figs. 7B, C, D). The fact that the semilunar rhythm appears at the time the tidal rhythm is entrained suggests that these rhythms are not separately but simultaneously entrained by the moonlight cycle.

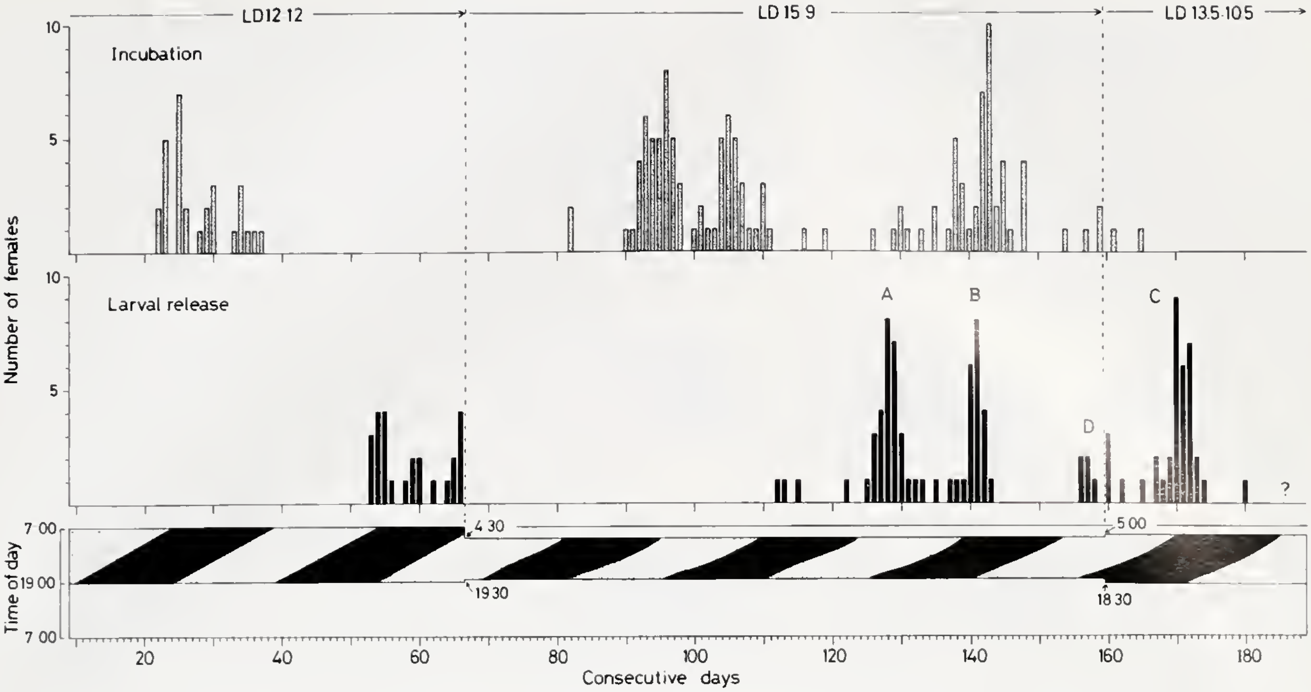


Figure 5(B). Plots of the number of females incubating a clutch and releasing larvae under simulated moonlight cycles (Seto population). Moonlight program applied to the animals is shown at the bottom of the figure (artificial moonlight is indicated by dotted area). The data of larval release were based on Figures 2A and B. Time of larval release was not monitored in a portion of females, so that the number of females releasing larvae per night does not precisely agree with the data shown in those figures. See text for further details.

All experiments reported above could not determine the process until individuals were synchronized by exposure to the artificial moonlight. One approach to this question is to apply the 24.5-h moonlight cycle to a freshly collected population showing a free-running tidal rhythm. For this purpose, ovigerous females (66) were collected at Kasaoka on 8 July 1986 and exposed to the artificial moonlight. In this experiment, the lights-off time in the 24-h LD cycle was set at 20:00. As the 'control' experiment, ovigerous females were collected at the same site on 12 August 1987 and maintained under the 24-h LD cycle alone (Fig. 8, right panel). If the animals were not exposed to artificial moonlight in this condition, *i.e.,* lights-off at 20:00, the larval release rhythm would free-run approximately in phase for 1–2 h delayed from the times of high water in the field (see Saigusa, 1986). On the other hand, entrainment to the artificial moonlight would be recognized when the phase relationship between the tidal rhythm and the moonlight cycle resembles those in Figures 2A and 2B. As Figure 8 (left panel) indicates, it took more than one month for the tidal rhythm to reach such a state after artificial moonlight was administered. Slopes of the new phase and variance of each activity were also similar to those recorded in Figure 2. A comparison of the data summarized in both panels (Fig. 8) represents a slight but clear discrepancy between the new phase and free-running phase in relation to the times of high tide. Therefore, it may be concluded that the left panel of Figure 8 shows a process where the tidal rhythm is phase-shifted and then entrained by the artificial moonlight. This process requires a long period even under the 24.5-h moonlight cycles.

Discussion

Data like those in Figures 2, 4, and 8 suggest that the females may have been responding to the retardation of the moonlight by 0.5 h to 0.8 h nightly. Previous data on entrainment of a semilunar rhythm (Saigusa, 1980) were similarly interpreted. However, retardation was not necessarily required to entrain the tidal and semilunar rhythms in Figures 6A and 7A. Ecological considerations may also favor the view that environmental stimuli such as moonrise and moonset are unsuitable synchronizing agents for the larval release rhythm of *Sesarma.* The intensity of moonlight would be extremely weak or nonexistent at the times of rise and fall of the moon even during the days of full moon. Furthermore, it is common that low clouds irregularly hide the moon at night. In such cases, it may be impossible for the animals to distin-

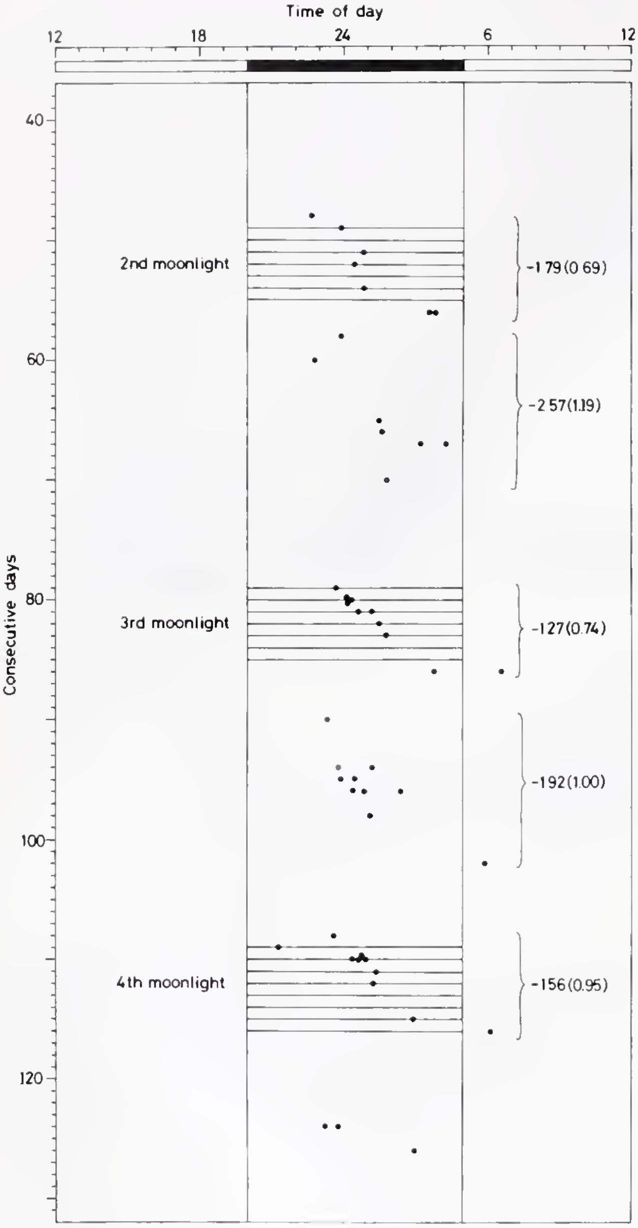
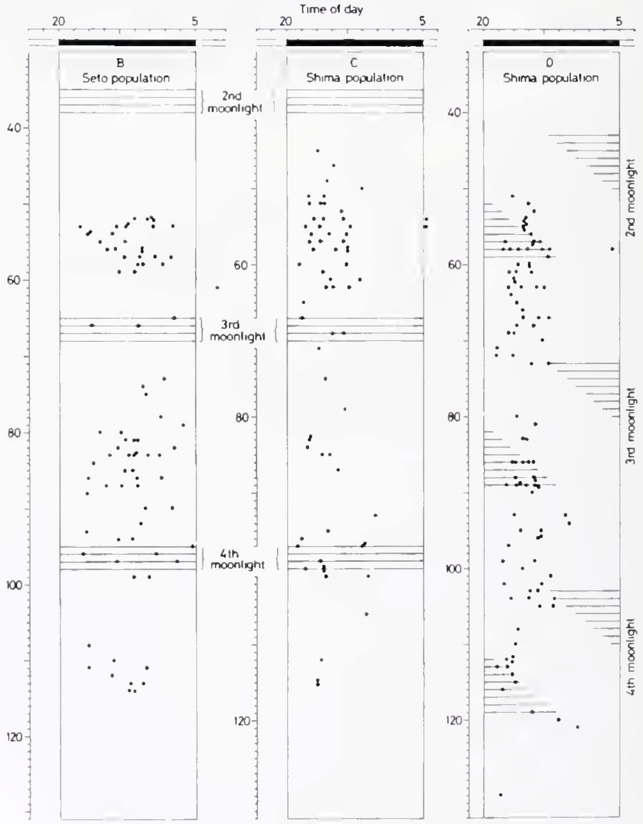


Figure 6(A). Time of day of larval release by the Seto population monitored under an artificial moonlight cycle. Moonlight is shown by the horizontal bars in the dark period of the 24-h LD cycle. Slope and variance of the activity pattern are shown on the right side of the corresponding data.

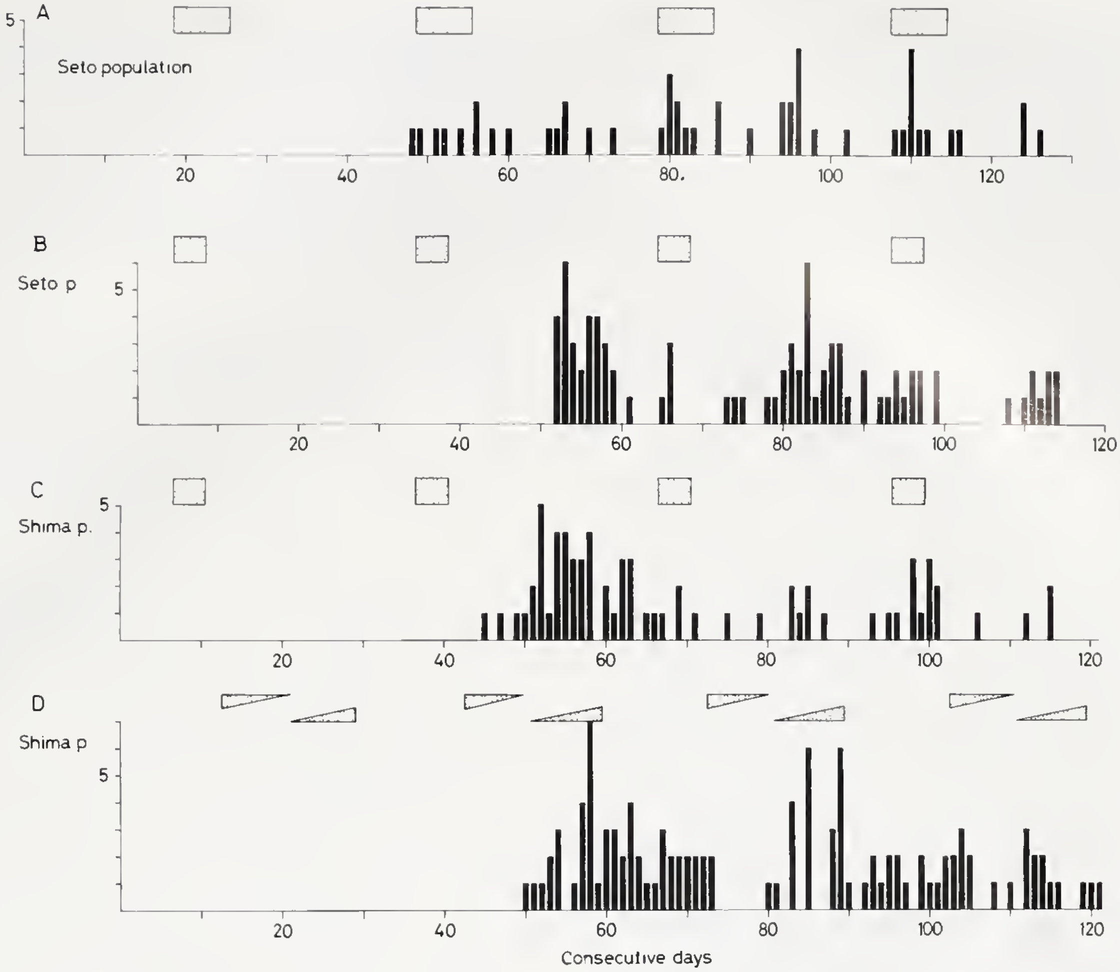
guish which is the real moonrise or moonset. This study certainly demonstrates that even a moonlight illumination for some days around the time of full moon functions for entrainment. But it could not determine the essential component for the entraining agent involved in moonlight cycles.

The pattern of the larval release rhythm of the Shima population (Figs. 4, 5A) differs from that of the Seto population (Figs. 2, 5B) as follows: (1) tidal component appears in the first half of the night; (2) the population has somewhat strong semilunar timing. Since the same zeitgeber cycles were given to both populations, such a difference in the pattern of the rhythm may be based on the properties specific to each population. The larval release pattern of the Shima population is clearly similar to that of the Izu population (Saigusa, 1985), which suggests that the pattern illustrated in Figure 4 is common to populations inhabiting the coast of the Pacific Ocean.

Another striking aspect on comparing the pattern of the tidal rhythm between Seto and Shima populations is different phase relations to the artificial moonlight cycles. Figure 9 represents times of high tide occurring at the seacoast near the habitats of each population. Kasaoka is located around the central part of the Seto Inland Sea, so that while the day-night and moonlight cycles differ by only 10 min between both areas, times of high tide are 4.5–5 h behind those at Shima. The degree of the phase difference between the evoked tidal rhythms in both populations clearly correspond to the time lag of the tidal cycles between these locations. Based on population-specific reactions to the moonlight cycle, this re-



Figures 6(B–D). Daily timing of larval release by Seto and Shima populations recorded under artificial moonlight cycles. Artificial moonlight is indicated by horizontal bars. Vertical axis: number of days after the animals were introduced to the laboratory.



Figures 7(A–D). Plots of the number of females releasing larvae per night (Seto and Shima populations). Artificial moonlight applied to the animals is indicated by the stippling on top of each data. The data are based on those of Figures 6A–D, respectively.

sult would give a sufficient explanation for synchronization of the daily timing of larval release with local tidal cycles.

Experiments on entrainment by simulated moonlight cycles, however, lead to a strong suspicion that local tidal cycles also can affect the timing of larval release. As field studies indicate, the pattern of *Sesarma* tidal rhythm is precisely synchronized with the times of high tide at night, especially for the Seto population (Saigusa, 1982). Hence, it seems that each female, at least for the Seto population, is capable of perceiving some information for precise timing from the tidal cycles near its habitats. Otherwise it would be impossible for this population to keep a precise phase relationship between the larval release rhythm and local tidal cycles over many generations. Nevertheless, possible entraining agents originating from tidal cycles are limited; the only remaining possibility was a periodic change in sound or vibration of surf which is carried to land (Saigusa, 1985). The activity pattern obtained under the artificial tidal cycles mimicking such stimuli, however, were less easily reconciled with tidal cycles as an entraining agent for endogenous timing (Saigusa, 1986). This might imply that such stimuli are too simple to function as a zeitgeber, otherwise they have no effect at least on internal timing.

Timing mechanisms underlying the circa-tidal rhythm of *Sesarma* have been described in terms of circadian systems (Saigusa, 1986). This model assumes two ‘circadian’ oscillators having a slightly different period to each other. The α-oscillation (*ca.* 24-h period), a so-called driving element, is subject to the environmental 24-h LD cycles. On the other hand, the β-oscillation, a driven element, is coupled to, and phased by, the α-oscillator. Larval release is linked to a phase of the β-oscillator. The period of this oscillator is about 24.5 h. (In my 1986 paper, it was described at about 24.8 h. However, because

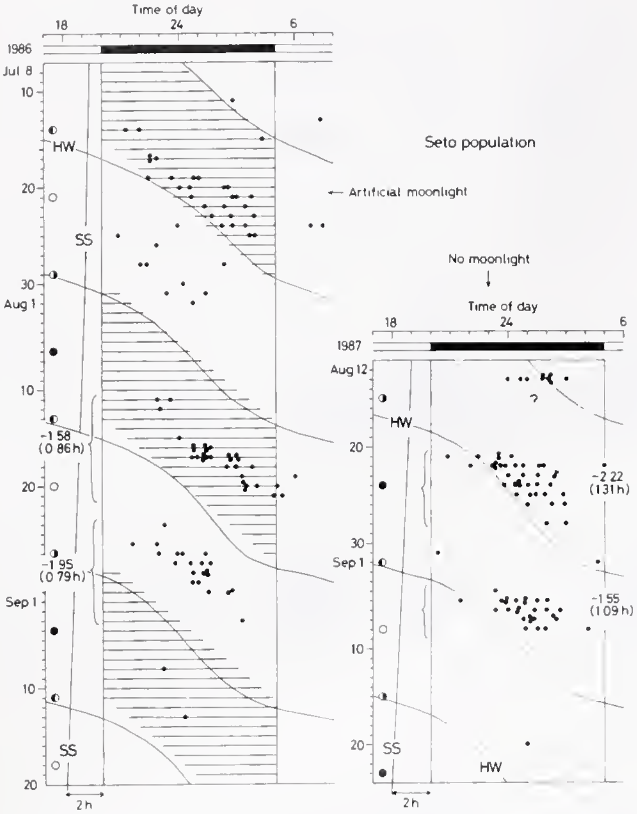


Figure 8. Time of day of larval release by the Seto population monitored under the 24.5-h moonlight cycle (left panel) and without moonlight (right panel). Diagonal lines (HW) connect the times of high water which should occur in the field at Kasaoka. *SS* connects the times of sunset in the field. Horizontal bars (left panel): artificial moonlight. Slope and variance in the activity pattern are presented on the left or right side of the corresponding data. In 1986 experiments these values were not calculated at the first half of the experimental period where no clear tidal component is seen an the activity pattern. Open circle, darkened circle, and semi-circles represent full moon, new moon, and the first or last quarters of the moon, respectively.

this oscillation follows the nocturnal high tide up to 7–8 h over the course of 15 days, it would be better to consider its period to be about 24.5 h in the Seto population. In the Shima population, slopes of the tidal rhythm were larger than those of the Seto population. This suggests that the period of β-oscillation is closer to that of the daily rhythm.) This hypothesis may be, indeed, an extention of the coupled circadian oscillator model which was developed for the daily rhythm of *Drosophila* eclosion (Pittendrigh and Bruce, 1959; Pittendrigh, 1960). But the important factor is the assumption that the driving oscillator postulated in *Sesarma* circa-tidal systems leads to the other oscillator which clearly differs in period, though slightly. This raises the critical question of what justification there is for such an assumption which has not been considered previously in circadian rhythms. In addition, the concept of 'oscillator' is not so specified as in Pittendrigh's model. For these reasons, *Sesarma*'s rhythm has been formulated in terms of α and β oscillators, not A and B oscillators.

According to this model, the data from Figures 1 and 3 regarding experiments in which the animals were kept for a long time under a 24-h LD cycle alone, is accounted for as a state in which the driven β-oscillator loses synchrony among individuals, though the α-oscillator is ap-

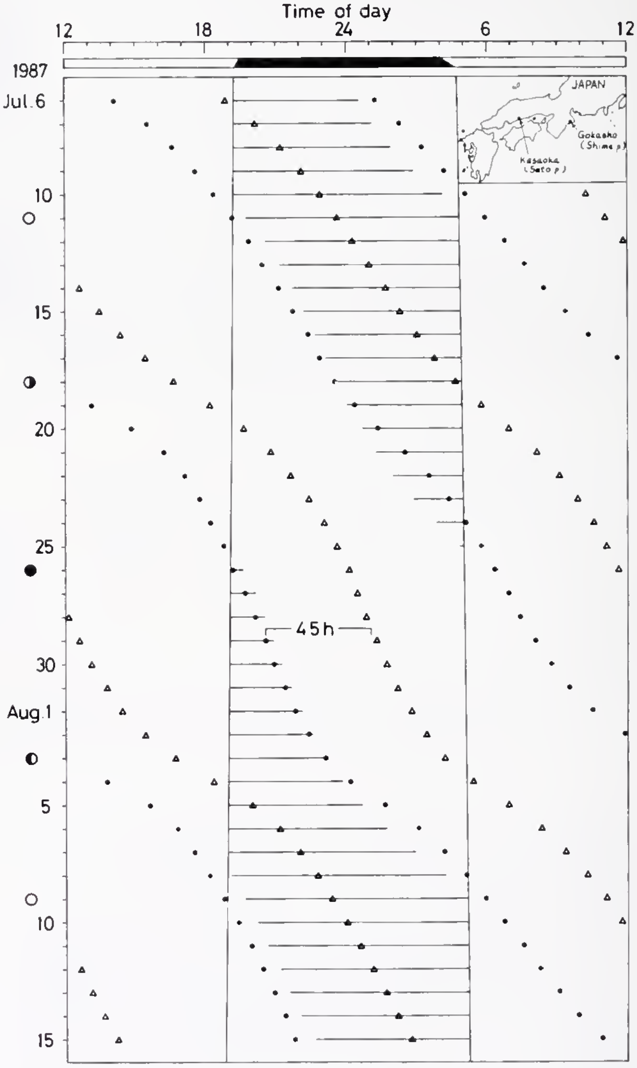


Figure 9. Plots of the times of high tide at the seacoasts of Gokasho (lat. 34°19″ N, log. 136°40″ E) and Kasaoka (lat. 34°30″ N, 133°30″ E). Day-night cycles (vertical lines) and natural moonlight cycles (horizontal bars) are drawn based on Gokasho data (the moonlight in the daytime is omitted). These environmental cycles are delayed only 10 min at Kasaoka. To avoid confusion, the data of Kasaoka are not illustrated. Dark circles and open triangles show the times of high tide occurring at Gokasho and Kasaoka, respectively. Phase relations between tidal and moonlight cycles in a certain location is about constant throughout a year; in this figure the data of July–August are summarized. Open circle, darkened circle, and semi-circle on the left side represent full moon, new moon, and half moon, respectively.

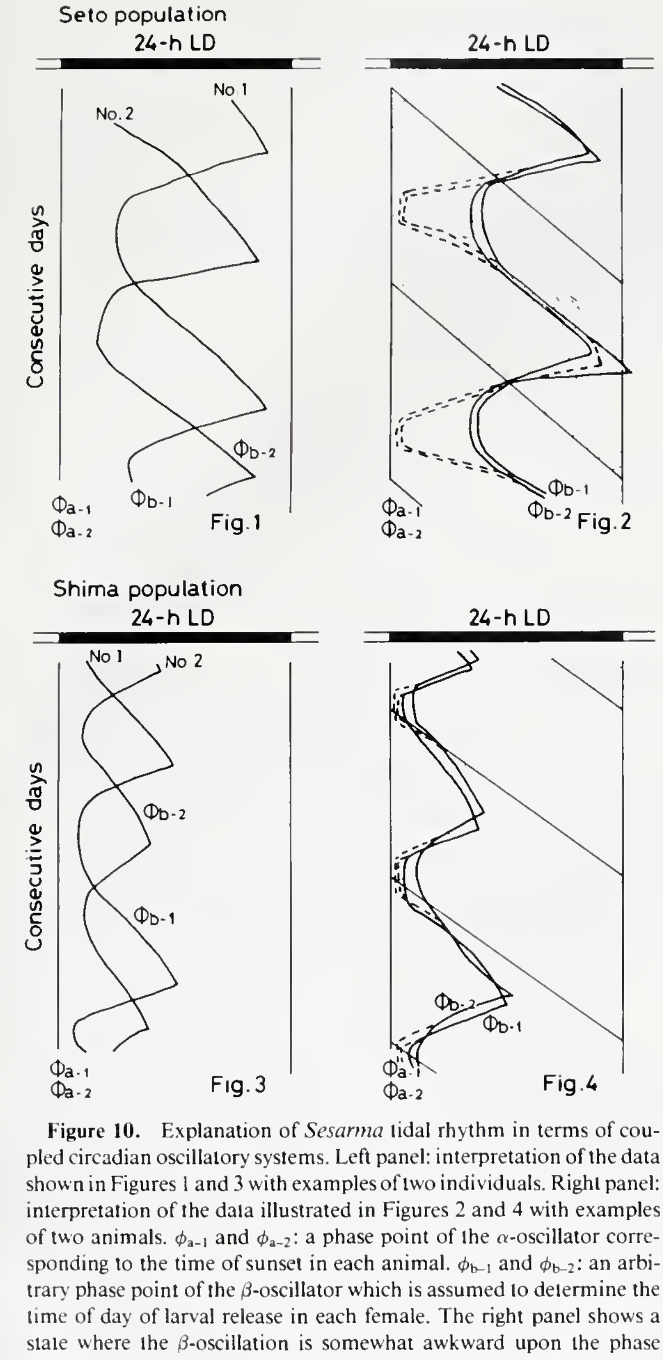


Figure 10. Explanation of *Sesarma* tidal rhythm in terms of coupled circadian oscillatory systems. Left panel: interpretation of the data shown in Figures 1 and 3 with examples of two individuals. Right panel: interpretation of the data illustrated in Figures 2 and 4 with examples of two animals. ϕ_{a-1} and ϕ_{a-2}: a phase point of the α-oscillator corresponding to the time of sunset in each animal. ϕ_{b-1} and ϕ_{b-2}: an arbitrary phase point of the β-oscillator which is assumed to determine the time of day of larval release in each female. The right panel shows a state where the β-oscillation is somewhat awkward upon the phase jump to the times of sunset in comparison to the natural conditions (the broken curves in the figure). Artificial moonlight is shown by dotted area.

parently synchronized with the light-dark cycle (Fig. 10, left panel). On the other hand, the data presented in Figures 2 and 4 provide obvious grounds for considering the phase of the β-oscillation to be coordinated by the moonlight cycle. A functional dichotomy between the driving and driven oscillators further suggests—in order to transform timing from being apparently arrhythmic to strongly coordinated—that the β-oscillator feeds back to the α-oscillator. In *Sesarma* circa-tidal rhythm, this process would require considerable time.

The tidal rhythm of *Sesarma* involves a phase jump around the first and last quarters of the moon (Saigusa, 1982, 1985). This phase jump was reproducible in constant conditions in the laboratory, which suggests that this timing is controlled endogenously (Saigusa, 1986). Field observations demonstrated that after the phase jump the timing of larval release is synchronized with dusk until the high water approaches night. As shown in Figure 2B, there was no clear indication of the activity coinciding with the light-off time in the 24-h LD cycle. In some cases the activity intruded into the light period. These laboratory observations indicate that the timing of the activity becomes somewhat awkward in its phase jump to the time of lights-off under the artificial conditions. If one accepts the reality of such a possibility, then the observed activity pattern would be explained in the manner shown in the right panel of Figure 10.

The main feature of the record illustrated in Figure 6A is that the tidal rhythm of *Sesarma* can be entrained by a moonlight cycle of one week occurring every 30 days. The question remains why a tidal component appeared in the activity pattern in the absence of either a 24.5-h or a 24.8-h component in the artificial moonlight cycles. The most obvious answer is that the circa-tidal rhythm of *Sesarma* itself involves an endogenous semi-monthly modulating component, which has been synchronized with the moonlight cycle. In other words, entrainment of a semilunar rhythm inevitably evokes a tidal rhythm in the timing systems of *Sesarma*.

Several workers (*e.g.*, Naylor, 1958; Bünning and Müller, 1961; Barnwell, 1968) have proposed that lunar and semilunar rhythms arise due to the occurrence of two separate rhythmic systems in an organism: one with a circadian period (12 h or 24 h), and another with a circa-tidal period (12.4 h or 24.8 h). Based on analogies of two physical oscillators, the superposition of these endogenous rhythms have been postulated to produce long-period rhythmicities as an additive effect in which the period differs slightly from each other. Field studies (Saigusa, 1982, 1985) certainly demonstrated that *Sesarma* tidal rhythm involves a daily component as well as a tidal component. However, on the basis of the response of this rhythm to 24-h LD cycles, there was no obvious ground for considering such an activity pattern to consist of an interaction of circadian and circa-tidal rhythms; a circadian rhythm with a period of 24.8 h was sufficient to account for the animal's tidally timed activity (Saigusa, 1986). Hence, the hypothesis that the pattern of the semilunar rhythm of *Sesarma* (Figs. 5A, B, 7A) is due to superposition of daily and tidal rhythms fails unambiguously.

Neumann (1975, 1981) proposed that the timing of emergence in the intertidal midge *Clunio* is controlled by two internal rhythmic systems in individual animals: a circa-semilunar rhythm related to the timing of pupation, and a circadian rhythm that determines the daily timing of emergence. He thought that, as a combination of these internal timing systems, emergence occurs at the time of low tide around the full and new moons.

The primary features of the larval release rhythm of *Sesarma* are as follows: (1) tidal and semilunar components are involved at the same time, and both recur at semi-monthly intervals; (2) the synchronizing agents of tidal and semilunar rhythms are the same; (3) tidal rhythm can be entrained by a moonlight cycle without the 24.5-h component; and (4) the semilunar component is detectable when the tidal component is clearly seen. These facts suggest that the tidal and semilunar rhythms of *Sesarma* do not exist separately in each animal, but must be identical. If one agrees with this viewpoint, then the pattern of the semilunar rhythm can be accounted for by the coupled circadian oscillatory systems mentioned above. In this case, endogenous semi-monthly component superimposed on the circa-tidal rhythm must actually be a function of the β-oscillation. The driven β-oscillator itself possesses a fortnightly amplitude modulation, the level of which affects the timing of egg-production and larval release possibly mediated by endocrine systems.

Acknowledgments

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Maternal Inhibition of Hatching at High Population Densities in *Tigriopus japonicus* (Copepoda, Crustacea)

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Abstract. A new mode of maternal protection is described for organisms that maintain contact with their developing embryos until hatching. Females of the copepod *Tigriopus japonicus* inhibit hatching of mature embryos (nauplii) from eggs they carry. Inhibition occurs at high population densities, or in the medium from crowded cultures. In contrast, when the mothers are killed or detached from their mature egg-sacs, all nauplii hatch within an hour, even in media from high-density cultures. A structure probably serving as an "umbilical cord" for transmission of the inhibitory message was demonstrated using electron microscopy.

Introduction

Population regulation by density-dependent mechanisms has been established for a wide variety of organisms (Peters and Barbosa, 1977; Stebbing and Heath, 1984), including copepods (Hicks and Coull, 1983)—crustacea that are ubiquitous in aquatic habitats. Progress in cultivation of harpacticoid copepods has resulted in high-density cultures for research and possibly aquaculture (Rothbard, 1976; Kahan, 1979, 1981; Kahan and Azoury, 1981; Chandler, 1986). In *Tigriopus japonicus* Mori (Mori, 1938; Ito, 1970)—a marine harpacticoid widely used in hatcheries in the Far East (Kuronuma and Fukusho, 1984) and reared in our laboratory—the percentage of females carrying mature egg-sacs increased in dense populations. This phenomenon, occurring under continuous or diurnal illumination, may signify a delay in hatching of mature embryos. Maternally induced arrest of embryonic development occurs in various organisms that lay eggs and release them (Gilbert, 1974; Clegg and Conte, 1980; Marcus, 1982; Yamashita and Hasegawa, 1985); it also occurs in certain mammals (Renfree, 1978). The present study is the first to describe delay in hatching of mature embryos from an egg-sac carried by the mother—a delay mediated by the living mother. Furthermore, a structure that resembles the "hooklets" described in another harpacticoid copepod (Fahrenbach, 1962) and that may serve as an "umbilical cord" transmitting the inhibitory message is shown.

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Materials and Methods

Tigriopus japonicus, (obtained from Ms. Huei-Mei Su Tseng, Tungkang Marine Laboratories, Taiwan) was cultivated in artificial seawater, salinity 35‰, prepared from Instant Ocean salts (Aquarium Systems, Mento, Ohio) in covered, rectangular, glass aquaria (30 × 16 × 20 cm) each containing 6 liters of medium. The cultures were maintained in a room at 20 ± 2°C with continuous lighting, hence permitting development of algae, and fed ad libitum on wheat germ. Like most harpacticoids, females of *T. japonicus* lay eggs in consecutive batches *i.e.*, egg-sacs, each attached to the abdomen. The emerging eggs (dark green) become orange-red during maturation. Two to three days pass between laying and the hatching of nauplii. In each experiment, performed under similar conditions, females carrying mature egg-sacs taken from the same high-density culture (5 [illegible] 10 per ml) were placed in conditioned or control medium at the densities specified, with a flake (about 0.5–1 mm) of wheat germ, in plastic cells 1.6 cm diameter (Multidish, Nunc, Denmark). Conditioned medium was obtained from the high-density cultures by removing the copepods and filtering the medium through filter paper. Filtered fresh medium, in which no organisms had been cultivated, was the control medium.

Hatching was observed under a dissecting microscope. All nauplii from the same egg-sac hatched almost simultaneously within a few minutes.

Effect of density on hatching

Because inhibition of hatching seemed likely in high-density cultures, the influence of population density was investigated first. Females carrying mature egg-sacs were taken from a dense culture and placed in a conditioned medium at varying densities: 40, 20, 10, 5, and 1 egg-carrying female(s) per ml. Hatching was observed for up to 48 hours. Sixty egg-carrying females comprised each experimental group.

Effect of conditioned medium on hatching

To ascertain the influence of conditioned media on hatching, single females carrying mature egg-sacs were maintained in 1 ml of either conditioned or control medium. Each experimental group comprised 48 females.

Effect of killing mothers on hatching

Does inhibition of hatching by conditioned medium act directly on the embryos, or indirectly, by way of the mother? To determine this, females carrying mature egg-sacs were killed by mechanical injury to the anterior cephalothorax, taking care not to harm the egg-sac. The females were then each transferred to 1 ml of either fresh or conditioned medium. Each experimental group had 48 egg-carrying females.

Effect of detachment from mother on hatching

Mature egg-sacs were detached as one unit, without harming the mother, by a method described earlier (Provasoli *et al.*, 1959; Betouhim-El and Kahan, 1972). Each egg-sac was then placed in 1 ml of conditioned medium. Sixty-two egg-sacs were observed. The above-mentioned experiments, presented graphically, were drawn on a Macintosh computer using Cricket graph software (Figs. 2–4).

Electron microscopy

For scanning and transmission electron microscopy, mature egg sac-carrying females were fixed in a solution containing 2.5% (w/v) glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.4, and 2.5% NaCl. For transmission electron microscopy, females were punctured immediately after transfer to the fixative to enhance penetration, left for three hours, then washed in cacodylate buffer 0.1 M, left overnight, and post-fixed with 1% (w/v) osmium tetroxide for 1 h. Dehydration was through graded ethanol solutions, then embedded in Spurr's medium. Thin sections were picked up on bare copper grids and stained in 1% (w/v) lead citrate for 5 min. Sections were examined in an AEJ electron microscope at 60 kV. For scanning electron microscopy, intact females were fixed, washed, and dehydrated as described above, dried by the critical point method, coated with gold-palladium, and viewed in a Jeol 840 scanning electron microscope.

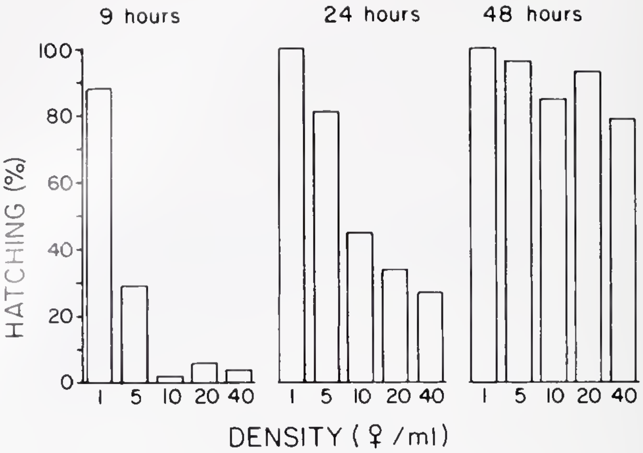


Figure 1. Effect of population density on hatching. Mature egg-sac-carrying females kept at the specified densities (1–40) in conditioned medium (see Materials and Methods). At each specified density the hatching percentage of 60 females was examined after 9, 24, and 48 hours.

Results

Effect of density on hatching

The percentage of nauplii hatching at the different experimental densities increased with time (Fig. 1). Hatching was observed in all groups after 9 hours. At the higher densities the hatching percentage was remarkably low, whereas at the lower densities (5 and 1) hatching percentage was higher (30 and 90%, respectively). After 24 hours, more mature egg-sacs had hatched in all the groups, revealing an inverse relationship between hatching percentage and population density. After 48 hours, hatching reached over 80% even at the highest density.

Effect of conditioned medium on hatching

The medium hatching time (time taken for hatching of the first 50% of the egg-sacs) in the conditioned medium was about 12 hours, whereas in the control (fresh medium) it was about 6 hours (Fig. 2).

Effect of killing mother on hatching

Figure 3 depicts the cumulative hatchings of egg-sacs remaining attached to recently killed females. Median

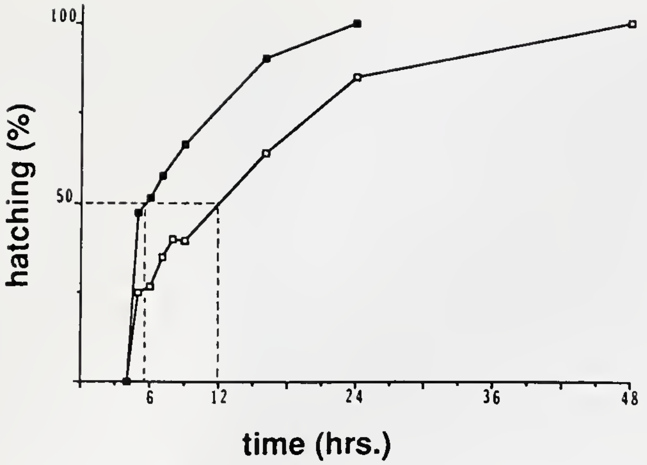


Figure 2. Effect of fresh (■ — ■) and conditioned medium (□ — □) on hatching of nauplii from mature egg-sacs carried by mothers isolated at density of 1/ml. Each experimental group consisted 48 females. Hatching percentage recorded as indicated, for a period to 48 hours. Dotted lines indicate time required for 50% of the females to hatch at each treatment.

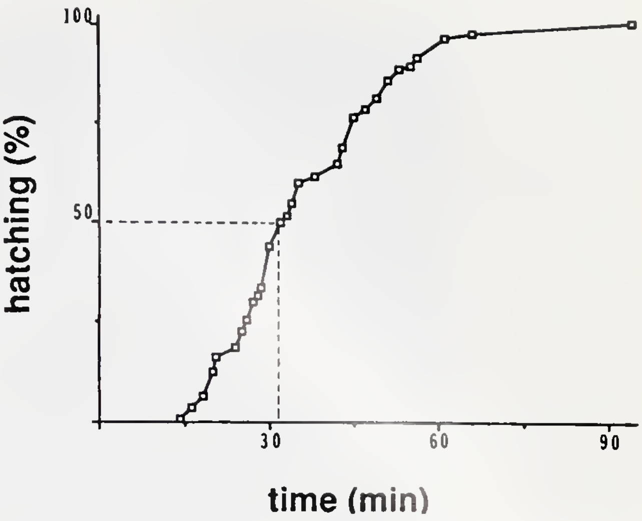


Figure 4. Hatching of nauplii from mature egg-sacs detached from the mother; 62 egg-sacs transferred. Each was placed in 1 ml conditioned medium. Dotted line indicates time for 50% of egg-sacs to hatch.

hatching time was less than a half-hour with conditioned as well as fresh medium.

Effect of detachment from mother on hatching

Figure 4 depicts the cumulative hatching of detached egg-sacs. The median hatching time resembled that for egg-sacs of killed mothers, *i.e.*, half an hour, even though the detached egg-sacs had been transferred to conditioned media.

Electron microscopy

Microscopy revealed a physical link between mother and egg-sac. Figure 5, a scanning electron micrograph, shows the ventral side of an egg-carrying female. In Figure 6, a lateral view of the abdomen of an egg-carrying female, one of the two connections are marked by an arrow. Figure 7, a scanning electron micrograph of a detached egg-sac, shows stubs of the two connections to the genitalia (arrows) on the anterior dorsal surface of the egg-sac. The transmission electron micrograph (Fig. 8) is a sagittal section through one of the two genital openings of the female, showing the connection.

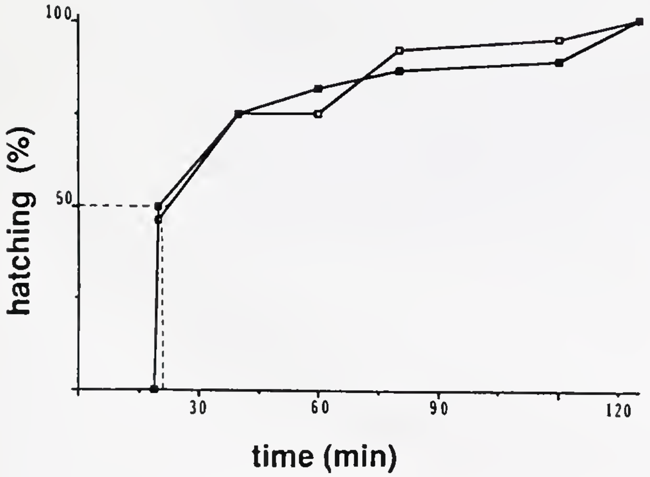
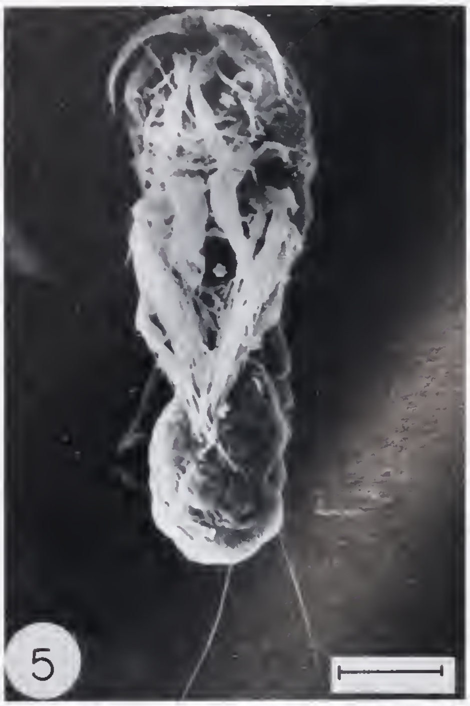


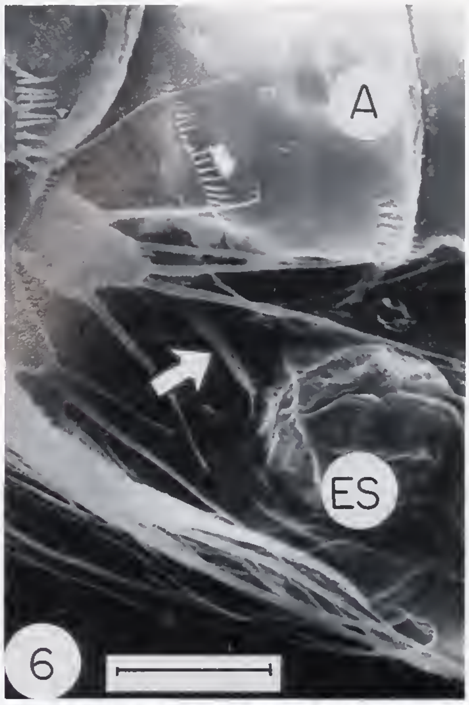
Figure 3. Effect of fresh (■ — ■) and conditioned medium (□ — □) on hatching of nauplii from mature egg-sacs carried by recently killed females; details as in Figure 2.

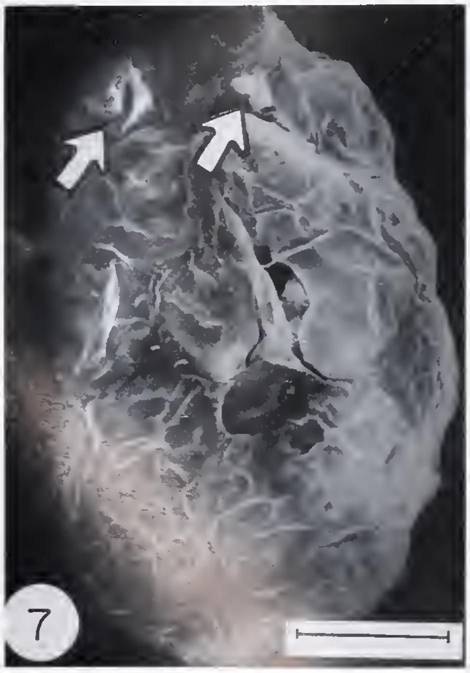
Discussion

The increased percentage of females carrying mature egg-sacs, first observed by us in dense cultures of *Tigriopus japonicus*, seemed to indicate a delay associated with high population density in the hatching of mature embryos. The results in Figures 1 and 2 confirm that high densities of egg-carrying females, or conditioned medium taken from dense cultures, tend to inhibit hatching, that effect diminishing considerably after 48 hours (Fig. 1).

The inhibitory effect probably is not exerted directly on the mature embryos, but rather, by the carrying mother as concluded from findings that mature embryos hatched rapidly from detached egg-sacs even after transfer to conditioned medium. Pr[illegible] *et al.* (1959) also noticed rapid hatching of detached mature egg-sacs from *T. japonicus* and *T. californicus*; they attributed this to nearness to an incandescent lamp. Evidence for the mother's involvement in the inhibitions was strengthened by the fact that mature egg-sacs attached to recently killed mothers hatched as rapidly as did detached egg sacs (Figs. 3, 4).







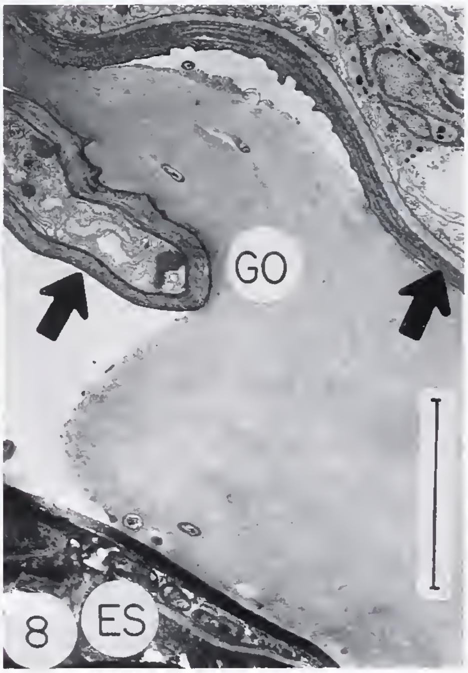


Figure 5. Scanning electron micrograph of an egg-carrying female. Ventral view. Bar: 200 μm.

Figure 6. Scanning electron micrograph of the abdomen of an egg-sac-carrying female (left lateral view); setae of appendages in foreground. Arrow indicates connection between abdomen of mother (A) and egg-sac (ES). Bar: 25 μm.

Figure 7. Scanning electron micrograph of detached egg-sac (dorsal view). Arrows indicate stubs of the two connections to the genitalia on the anterior surface. Bar: 50 μm.

Figure 8. Transmission electron micrograph of connection between genital opening (GO) and egg-sac (ES). Sagittal section; arrows indicate abdominal cuticle. Bar: 5 μm.

Injury to or removal of the head triggers egg-laying in many invertebrates, therefore attributed to annullment of a cerebrally controlled inhibition (Adiyodi and Adiyodi, 1983). But in *T. japonicus,* hatching of mature egg-sacs is evidently not triggered by their being pushed out—detached—by laying down of a new egg-sac. Females killed by mechanical injury to the cephalothorax did not lay down a new egg-sac, and the nauplii hatched while the egg-sac remained attached to the mother. Moreover, nauplii normally hatch from the egg-sac while it is carried by the female; the newly laid egg-sac appears only after the hatching of the mature one. Walker (1979) also noted that in the marine harpacticoid copepod *Amphiascoides,* a new paired sac forms only upon release of the former pair.

The live mother also mediates recovery from the inhibitory effect of crowding as inferred by comparing Figures 2 and 3, derived from experiments with copepods from the same culture and performed under identical conditions. For egg-sacs carried by a recently killed mother, the inhibition is annulled quickly. The median hatching time is within a half-hour (Fig. 3). But when they are carried by living mothers, inhibition continues for a long time, even following transfer to fresh medium. The median hatching time is about 6 hours (Fig. 2).

Inhibition depends on some sort of connection between mother and mature embryos: mere proximity of the unharmed mother to her detached egg-sac did not prevent early hatching; when only part of an egg-sac was detached, all eggs in the detached portion hatched quickly. Those remaining in the attached portion hatched much later (preliminary results). The structure shown in Figures 6–8 may serve as a conduit between the mother and her egg-sac. Accordingly, we postulate that inhibition of hatching in mature embryos is controlled by the mother through an "umbilical cord"-mechanism triggered by adverse conditions, *e.g.*, crowding. It reveals a new form of maternal care in organisms that maintain contact with their eggs until hatching. The delay of hatching of mature embryos found in *T. japonicus* may be analogous to developmental arrest in mammals (Renfree, 1978), which also maintain contact with their developing embryos.

Another type of maternal involvement occurs in other crustaceans *e.g.*, notodelphyoid copepods (Davis, 1968) and the crab *Rhithropanopeus harrisii* (Forward and Lohmann, 1983). In the copepods the mother's movements help nauplii to emerge; in the crab, a chemical cue released by the first larvae triggers the mother's aid in the hatching of the remaining brood. In cirripeds, a small amount of the mother's hemolymph promotes hatching of mature eggs (Crisp, 1956, 1969; Crisp and Spencer, 1958). Enhanced hatching in *Tigriopus* (Figs. 3, 4) may be triggered by a substance released from an unnoticed wound inflicted on the mother while the egg-sac was detaching. This possibility was eliminated, as no enhanced hatching was noticed, in a preliminary experiment wherein homogenates of *Tigriopus* mothers were added to the medium where mature egg sac-carrying females were maintained. Maternal control of hatching in the aforementioned various crustaceans is thus promotive. It is inhibitory in *Tigriopus.*

A reproductive strategy involving delay in the hatching of nearly mature embryos from eggs laid and no longer in contact with the mother occurs in the squid *Loligo vulgaris* (Marthy *et al.*, 1976; Weischer and Marthy, 1983). Delay is caused by a tranquilizer in the perivitelline fluid of the eggs. However, a natural tranquilizer seems uninvolved in inhibition of hatching in *T. japonicus* since a macerate of mature egg-carrying females (40 per ml) did not tranquilize nauplii.

The maternally inhibited hatching mechanism described here might be advantageous for *T. japonicus* in its tide-pool habitat, which is characterized by diurnal short-period fluctuations. Igarashi (1959) did find wide fluctuations in population density and age composition of *T. japonicus* in various kinds of tidal pools. When tidal pools are densely populated, the inhibitory mechanism could delay hatching of offspring until the next tide distributed the nauplii to less crowded habitats. The inhibitory period of up to 48 hours found in the highest densities of females tested (Fig. 1) could also be advantageous for populations in tidal pools where fluctuations are less frequent *e.g.*, pools located at higher tidal levels. This delayed mechanism alone, or along with others found in various copepods under crowded culture conditions [*e.g.*, changes in age distribution, sex ratio, fertile period, number of ovisacs and eggs (Hicks and Coull 1983; Walker, 1979; Kahan and Azoury 1981; Kahan *et al.*, unpubl.)] might regulate population growth in *T. japonicus.* Whether the regulation in *Tigriopus* is common in other copepods and aquatic organisms remains to be determined. A better understanding of hatching in aquatic invertebrates, urged by Davis (1981), should help elucidate the mechanism of maternally controlled hatching in *T. japonicus.*

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Initial Evidence for the Transport of Teleplanic Larvae of Benthic Invertebrates Across the East Pacific Barrier

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> Here is the great immeasurable sea,
> in which move creatures beyond number.
> *Jerusalem Bible,* Psalm 104:25.

Abstract. Since the mid-19th century biologists have considered the east tropical Pacific to be a barrier for the dispersal of coastal marine invertebrate species. More recently it has been maintained that this is so because planktonic larvae are unable to cross such a large expanse of ocean. Therefore, it seems extraordinary that no observations have been made to determine whether larvae of invertebrates are actually transported by the major currents of that region. Plankton samples in the present study show that invertebrate larvae do occur within the east tropical Pacific including, but not restricted to, those of gastropods, polychaetes, sipunculans, decapod crustacea, echinoderms, and coelenterates—though as a rule, their occurrence there is significantly less than within the central tropical Pacific.

Data from larval distributions suggest that the east tropical Pacific may act as a substantial impediment to many invertebrate forms, but that it is not a complete barrier to dispersal. Accordingly, the region is best considered a filter. It allows only species with a potential for an exceptionally long larval life to pass *i.e.,* those with teleplanic larvae, while it blocks other forms that are restricted to a shorter time in the plankton owing to an inability to delay metamorphosis or lack of an alternate mode of dispersal. The capacity for dispersal by planktonic larvae differs among the various taxa.

Introduction

There lies within the region bounded to the north and south by the tropics of Cancer and Capricorn, to the west by the Polynesian Islands, and to the east by the Pacific coastline of continental America (*i.e.,* Mexico, Central and northwest South America) and its adjacent islands, the Clipperton, Cocos, Revillagigedo, and Galapagos archipelagos—a vast area of tropical sea devoid of oceanic islands and known to biogeographers as the "east Pacific barrier" (Ekman, 1953) (Fig. 1). Darwin (1860) referred to this wide expanse of open ocean as an "impassable barrier" for the migration of coastal marine species. One hundred years later Thorson (1961) declared that "under average conditions even 'long distance' larvae have a much too short pelagic life to survive the critical distance across the eastern Pacific." Subsequently, other biogeographers have not been so sure. Garth (1965, 1974) found that approximately 50 percent of the brachyuran crabs from the Clipperton Islands in the eastern Pacific were of Indo-Pacific origin and at least 7 Indo-Pacific decapod species found commensal with hermatypic corals were commonly recorded in tropical east Pacific reefs. He attributed this distribution to the dispersal of larvae across the "east Pacific barrier" (Garth, 1966). Briggs (1974) concluded that there "is a one way filter" and that it is possible to speculate that competitively dominant species continue to migrate, as they probably have for a long time, from the Indo-west Pacific eastward across the open ocean to America. . . ." V[illegible] (1978, pp. 253–257) believed from indirect evidence that the one-way influx of Indo-west Pacific species into the eastern tropical Pacific "which may have begun in the Pliocene" was largely the result of dispersal of "long-lived teleplanic larvae," but supposed that biological and physical factors very likely are also important for the success or failure of

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This paper is for Amélie, helpmate, friend, and colleague in celebration of our one-third century of marriage—October 4, 1988.

emigration to the American continent. On speculating upon the possibility of passive transport of larvae across the east tropical Pacific, Zinsmeister and Emerson (1979) concluded that such dispersal is possible but that the paucity of Indo-Pacific molluscan species is also the consequence of the "vast expanse of open water" and the "lack of suitable habitats with available ecological niches. . . ." Leis (1984) maintained that the hypothesis of larval dispersal across the "east Pacific barrier" must finally hinge upon the demonstration that larvae actually occur within currents of the tropical east Pacific and allowed that "unequivocal crossings of a major portion of the barrier have not been documented." Although there has been much conjecture, the transport of larvae across the "east Pacific barrier" has yet to be demonstrated; indeed it seems extraordinary that over the past century no previous attempt has been made to determine whether any larvae of benthic invertebrates can actually be found in currents of the tropical east Pacific Ocean.

Accordingly, the research described here addresses two fundamental questions. First, is there any evidence that larvae belonging to nearshore species actually occur in the plankton within the region of the "east Pacific barrier"? Second, if so, what invertebrate taxa are represented and is there evidence for differences in the capacity for dispersal among taxa to cross this barrier?

Material and Methods

Plankton samples from the tropical east Pacific were examined from the locations indicated on Figure 1. Samples were taken from collections at the Scripps Institution of Oceanography, including those of the Capricorn Expedition (1952–53), the Eastropac Expedition (1967), and the Domes Expedition (1975–76). Samples were also collected for the present study during: the 73rd scientific cruise of the R.V. KNORR (1979) from the region near Hawaii, the cruise of R.V. THOMAS WASHINGTON on the 3rd leg of the Papatua Expedition (1985) between Mexico and the Samoa Islands, and most recently the Helios Expedition (1987) on the R.V. MELVILLE between San Diego and Tahiti. Most samples were taken obliquely from depths between 150 to 200 meters and the surface, using three-quarter meter diameter and 240 to 360 μm mesh nets. Two hundred and nineteen samples from the tropical eastern Pacific were examined for the larvae of sublittoral benthic species.

Results

Two major currents may transport larvae eastward across the east tropical Pacific Ocean: the Equatorial Countercurrent and Equatorial Undercurrent (Fig. 1; see also Wyrtki, 1965). If transported from west to east, larvae of invertebrates should occur within these currents. Plankton samples reveal that teleplanic larvae are indeed found within these major equatorial currents. Thus, dispersal within the region of the tropical east Pacific is not only possible but must actually occur. However, the presence of larvae does not prove that the east Pacific barrier is successfully crossed; only faunal data can ultimately show this.

The kinds of larvae encountered encompass a wide spectrum of invertebrate taxa. Samples from within the tropical east Pacific included but were not restricted to (a) veligers of benthic gastropods and bivalves; (b) chaetosphaera, mesotrocha, and other polychaete larval types; (c) pelagosphaera of sipunculans; (d) zoea and megalopa of brachyuran decapoda; (e) plutei, bipinnaria, and brachiolaria of echinoderms; and (f) the Semper's cerianthula and planula larvae of coelenterates.

Not all invertebrate groups are equally represented. One example has been selected to illustrate apparent differences that occur in the dispersal capacity among major taxa, at least insofar as this may be reflected by the frequency at which their larvae are encountered in the plankton (Fig. 2).

Gastropod veligers (Fig. 2A) were found in approximately 42% of localities sampled in the eastern tropical Pacific Ocean (as defined in the footnote in Table I) and are represented largely by families already known to have teleplanic larvae (Scheltema, 1986a), *e.g.*, the Architectonicidae (Fig. 3A, B) and Cymatiidae (= Ranellidae). In contrast, polychaete larvae were found at only 28% of all localities sampled (Fig. 2B; 3C–F) and only seldom in the central region of the tropical east Pacific. For instance, only a few teleplanic larvae of the otherwise commonly found polychaete families, Spionidae and Chaetopteridae, are encountered between 120° and 140°W longitude whereas in the central Pacific these families are represented at more than half of the locations sampled (Scheltema, 1986a). Past studies in the Atlantic show that teleplanic larvae in general and the families of gastropods and polychaetes considered here in particular show no discernible seasonal periodicity in their occurrence in the open sea, and that therefore the relative frequency at which larvae are found may be considered as a first approximation a measure of the capacity for long-distance dispersal. If frequency of occurrence is a valid criterion, then there is likely to be a significant difference between gastropods and polychaetes in their capacity for transport across the east tropical Pacific.

The more general question may now be asked: To what extent is the east tropical Pacific likely to be a barrier to dispersal? To gain some insight into this question, one can compare the relative frequency that major taxa occur in the east with that of the central tropical Pacific (Table I). Larvae of decapods, polychaetes, sipunculans, and gastropods occur at significantly lesser frequencies in

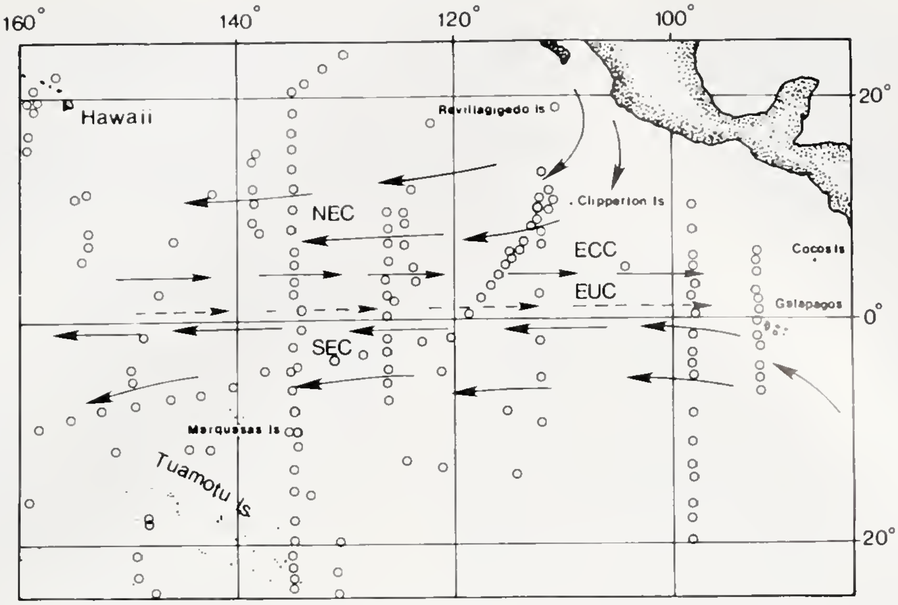

Figure 1. Distribution of sampling locations in relation to generalized surface and near surface currents of the eastern tropical Pacific Ocean. *Open circles* indicate the positions where oblique plankton samples were taken. *Arrows* illustrate generalized patterns of major surface circulation. *Currents from west to east:* Equatorial Undercurrent (EUC) shown by dashed line occurs between 50 and approximately 300 meters depth; the Equatorial Countercurrent (ECC) lies between the surface and approximately 50 meters depth. The Countercurrent is well developed between August and December and absent during February to April. *Currents from east to west:* North Equatorial Current (NEC); South Equatorial Current (SEC). Details of the strength and direction of currents are very much more complex than shown and are subject to seasonal variations (see Wyrtki 1965, 1966 for a detailed discussion).

equal areas of the east than in the central tropical Pacific (compare column 2 and 4, Table I). On the other hand, the Semper's, cerianthula, and planula larvae of coelenterates evidently are not significantly impeded by the "east Pacific barrier." There are data indicating a greater incidence of echinoderm larvae in the east (47%) than in the central tropical Pacific (16%) (not shown in Table I). This is misleading, since larval skeletons in the older central Pacific samples—some preserved over twenty years—will have deteriorated with time resulting in an underestimate of pluteus abundance (see Scheltema, 1986a; p. 243 for source of central Pacific samples). The table shows that overall the east Pacific may act as a substantial barrier to many forms. The data further suggest that the east Pacific does act as a filter and that it is a significant but incomplete barrier to larval dispersal.

Discussion

Three kinds of evidence can be used to support the hypothesis that larvae may contribute to the spread and maintenance of the Indo-Pacific species across the "east Pacific barrier." Evidence of the actual presence or absence of larvae in the major ocean currents alone may be insufficient because although it admits the possibility, such information says nothing about the actual success of larval dispersal and its relationship to species ranges. Indirect evidence from the geographical distribution of species is also required to show that the range of an Indo-Pacific form has actually extended into the eastern Pacific. A third kind of evidence indicating that larvae are successfully transported across the east Pacific may come from a comparison of genetic similarity between populations of Indo-Pacific species common to both the central and eastern Pacific. Taken together these three kinds of evidence can allow reasonable inferences to be made regarding larval dispersal of Indo-Pacific species into the eastern Pacific and the likelihood that continuing exchange of larvae in both directions maintains genetic continuity. Separately, each form of evidence has weaknesses and cannot provide a complete answer.

Although the presence of larvae in the plankton provides evidence that dispersal actually happens, their absence cannot prove that it does not occur. The spatial and temporal occurrence of larvae may be such that they go undetected. Larvae that occur at concentrations of less than 1.35×10^{-3} m^{-3} will not be captured in every twenty-minute tow, yet such concentrations probably are sufficient to act effectively as agents of genetic ex-

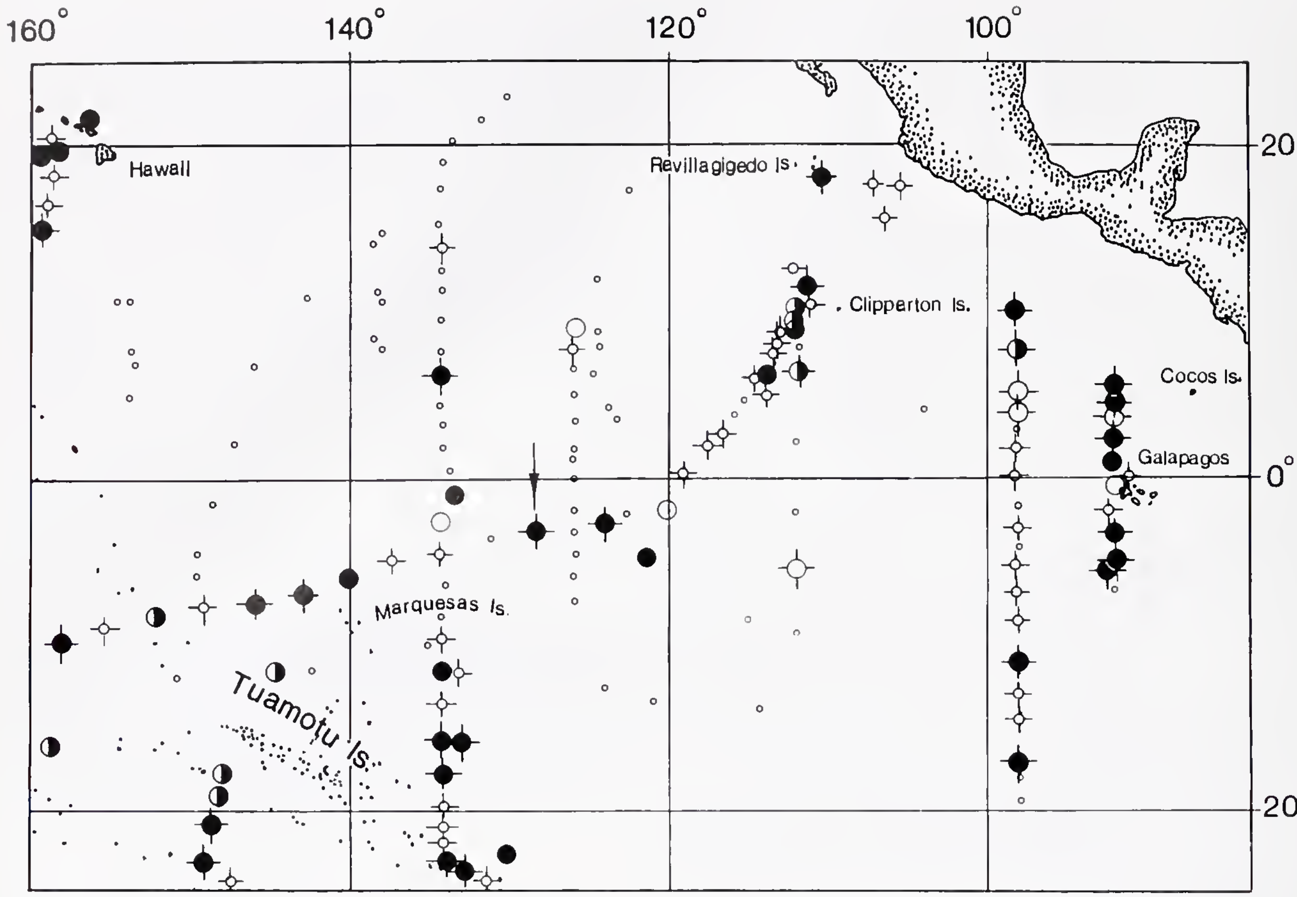

Figure 2A, B. Distribution of teleplanic larvae of coastal shoal-water invertebrates encountered at 219 locations in the open waters of the "east Pacific barrier." A. Locations where veliger larvae of gastropod molluscs were found. *Large filled circles* denote presence of veligers belonging to the family Architectonicidae (see Fig. 3A, B); *large open circles* signify localities where Cymatiidae (= Ranellidae) were recovered; *large half-filled circles* show positions where the larvae of both Architectonicidae and Cymatiidae were encountered; *smaller circles* with rays designate veligers other than those of the two commonmost families of gastropods. *Rays on large circles* indicate that other gastropod veligers were also found. *Minute circles* indicate negative stations where no gastropod veligers were recovered. *Arrow* shows where specimen in Figure 3A and B was collected.

change (Scheltema, 1971; p. 313) and under some circumstances for colonization. Only positive data on the occurrence of larvae are useful; negative data must always remain equivocal. Another familiar source of error may be avoidance of the net as probably occurs among some decapod crustacean larvae.

To ascertain whether larvae of Indo-Pacific species found in the plankton have been effective in colonizing eastern Pacific islands or the American continent, the geographic range of species must be known. In particular it should be demonstrated that the Indo-Pacific species found in the eastern Pacific have planktonic larvae. Return now to the two examples previously used. Among the gastropods Emerson (1982; 1983) reported 49 species of Indo-Pacific prosobranch gastropods in the eastern Pacific. Among these, 38% are known from the mainland while the remaining 62% are found only on islands (32 species on Clipperton, 3 on Cocos, 9 on the Galapagos, and 1 on Guadalupe). Additional species are attributed by Vermeij (1978) and Shasky (1985). Between 68 and 75 species of Indo-Pacific affinity are now known in the eastern tropical Pacific Ocean. Each year new species are added to the list but the percentage will always remain relatively small (perhaps between 2–3%) compared to the total eastern tropical Pacific gastropod fauna (see Keen, 1971). Most of those species cited by Emerson (1978) are either known to have teleplanic larvae or, if the mode of development is unknown, then belong to families that commonly do. In a study of the polychaetes from Panama, Fauchald (1977) reported 3% (7 spp.) of Indo-Pacific warm-water origin, but his study was restricted to sandy intertidal regions. There were also 28 species re-

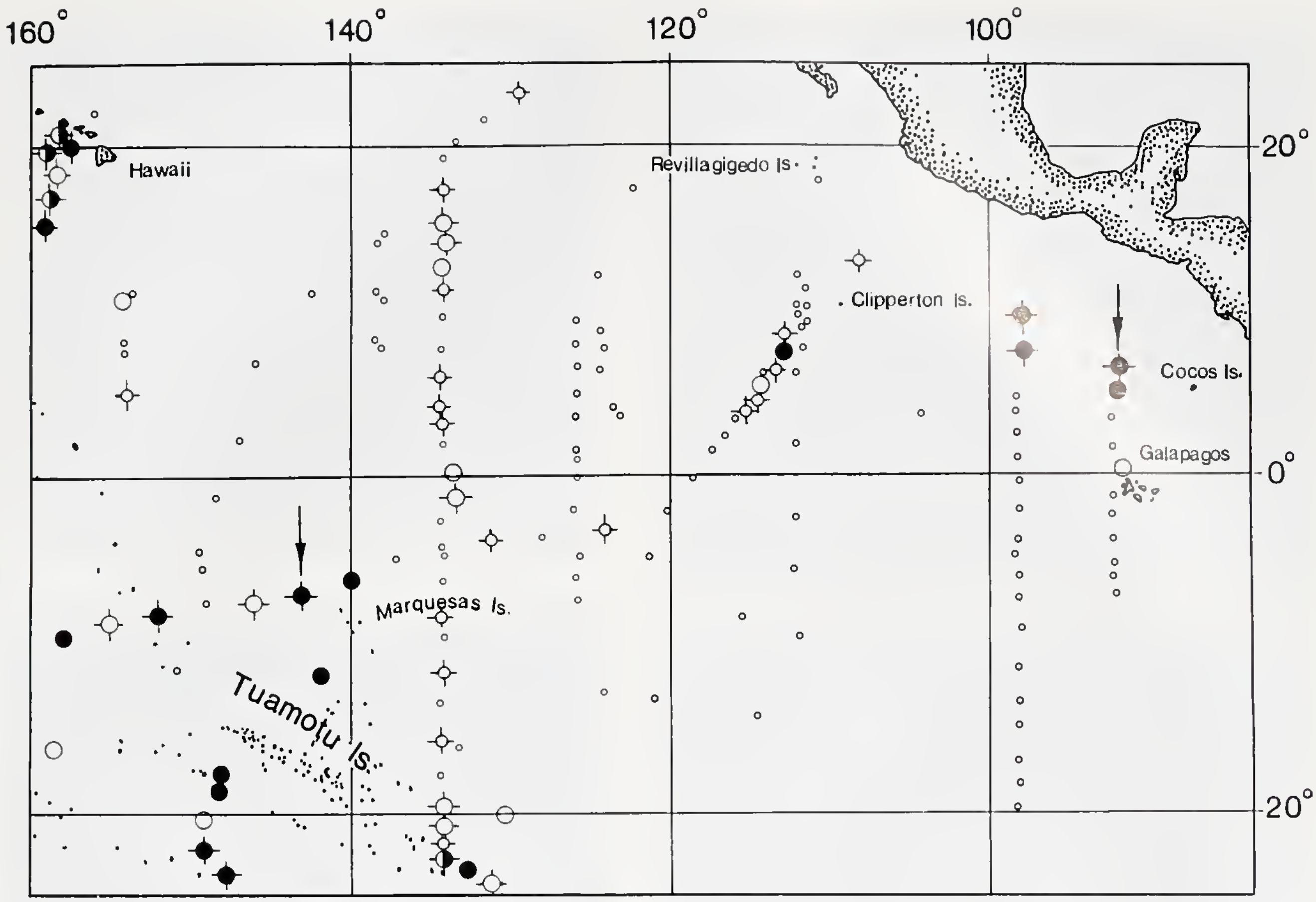

Figure 2. B. Locations where larvae of polychaetes were encountered. *Large filled circles* show where chaetosphaera larvae of spionids were located (see Fig. 3C, D, E, F); *large open circles* denote localities where mesotroch larvae of Chaetopteridae were found; *half-filled circles* show positions where both Spionidae and Chaetopteridae were found. *Smaller circles* with rays denote localities where polychaete larvae other than the two most commonly represented families occurred. *Rays on large circles* indicate presence of other polychaete larvae in addition to spionids and chaetopterids. *Minute circles* indicate localities where polychaete larvae were absent in the samples. *Arrows* show points of collection of specimens in Figure 3C, D, E, F.

garded as circumtropical whose wide geographic distribution may be the result of larval dispersal. Since the study was of a restricted habitat, one must consider the data with great reservation. The current knowledge of eastern Pacific polychaetes is probably insufficient to make any broad generalization.

Finally a third kind of evidence, measurement of genetic similarities or differences between allopatric populations, has scarcely been attempted for benthic invertebrate species. Marked genetic similarity between central and eastern tropical Pacific populations of species would suggest sufficient gene flow to maintain the integrity of widely distributed forms. Huber (1985) showed that such gene flow probably occurs between Hawaiian and Panamanian populations of the brachyuran decapod crustacean *Trapezia ferruginea*, since electrophoretic data show no genetic distance between them. To maintain such similarity, larval or some other mode of dispersal such as rafting (Scheltema, 1986b) would be required, *i.e.*, dispersal is a necessary if not a sufficient requirement for genetic exchange. The potential for such an approach has been further suggested by Rosenblatt and Waples (1986). They showed that populations of 12 species of "trans-Pacific" shore fish (*i.e.*, from Hawaii and the eastern Pacific) are more similar genetically than populations of some of the same species from either side of the Isthmus of Panama.

Although the data on larval [illegible] in the east tropical Pacific generally support the hypothesis that dispersal across the "east Pacific barrier" is possible at least for some species, another perplexing problem remains. Is the exchange of larvae completed in only one direction? Most biogeographical studies give evidence for a persistent but small faunal element of Indo-Pacific

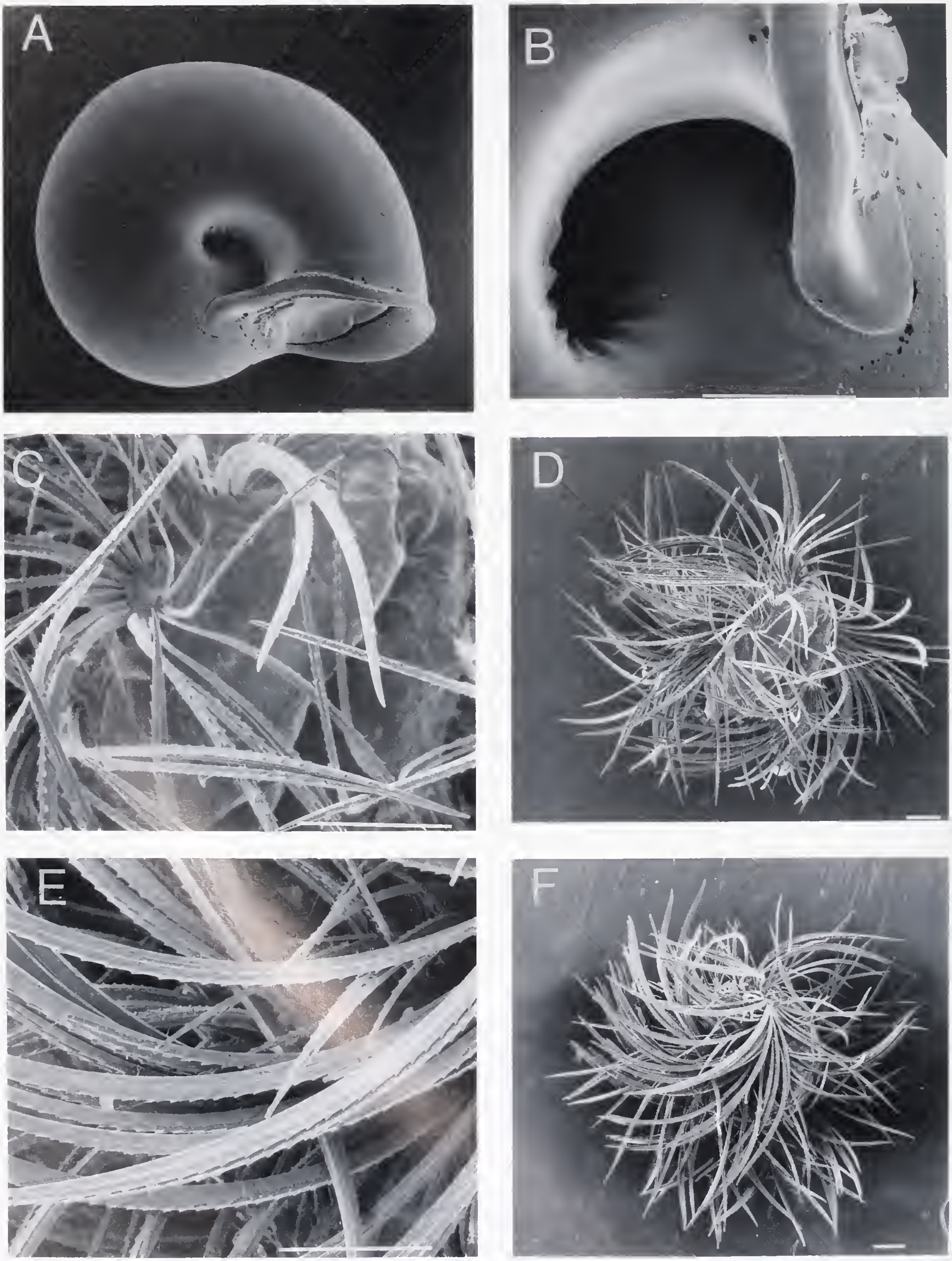
A
B
C
D
E
F

Table I

Differences in the frequency at which the larvae of benthic invertebrates occur in equal areas of the east and central tropical Pacific

	East Pacific Barrier* (total no. sta. = 100)		Central Pacific** (total no. sta. = 129)		
	No. sta. (1)	% sta. (2)	No. sta. (3)	% sta. (4)	*P****
Decapoda (zoea, megalopa)	9	9	91	71	0.001
Polychaeta (chaetosphaera, mesotrocha, mitraria, etc.)	28	28	77	60	0.001
Sipuncula (pelagosphaera)	33	33	100	78	0.001
Gastropoda (veligers)	42	41	91	71	0.001
Coelenterates (Semper's cerianthula planula)	66	66	70	54	n.s.

* Tropical east Pacific barrier = 100°W–140°W; 23.5°N–23.5°S.

** Central tropical Pacific = 160°E–160°W; 23.5°N–23.5°S.

*** Value of *P* for ^{2} test of homogeneity (comparison between values in column 2 and 4).

affinity in the coastal east tropical Pacific but none for a complimentary east Pacific fauna in the central or Indo-Pacific (see Briggs, 1974; Vermeij, 1978). The assertion by Zinsmeister and Emerson (1979) that "the absence of warm westward flowing currents has prevented Panamic species with long pelagic stages from reaching the central Pacific" must be mistaken. At least during part of the year, both the westward flowing North and South Equatorial Currents (Fig. 1) have surface temperatures exceeding 26°C. Moreover, larvae of tropical species are actually found in both of these major currents. Consequently, these larvae must be dispersed from east to west (Fig. 2). Temperatures from the surface to the first 50 meters of the North and South Equatorial Currents are well within the range tolerated by tropical teleplanic larvae. The misconception of Zinsmeister and Emerson (1979) may arise from the erroneous notion that the westward flowing North and South Equatorial Currents originate entirely from the California and Peru Currents, respectively. However, Wyrtki (1966, p. 62, Fig. 10) notes that between the months of August and December, when it is well developed, the warm countercurrent may, upon approaching the South American continent, contribute up to 37% of the total water mass of the North Equatorial Current. The combined contributions of the countercurrent and undercurrent may somewhat exceed half of the total water mass transported by the North Equatorial Current. Likewise, the countercurrent and undercurrent contribute to the South Equatorial Current during this time of year. Wyrtki (1965, p. 274) further concludes that between August and December "the water of the Costa Rica Coastal Current passes directly into the North Equatorial Current." This current, it is supposed, will provide a possible conduit for larvae from the coast of Central America to the North Equatorial Current. However between February and April the countercurrent disappears entirely. Thus, it is obvious that the current system in the easternmost Pacific has marked temporal and spatial variability making predictions about the transport of invertebrate larvae very difficult. The phenomenon of El Niño can add still further variability such as changes in speed and direction of surface currents as well as increase in the surface temperature. Such large fluctuations may provide further opportunities for dispersal of invertebrate larvae which may be unavailable during average conditions.

Figure 3. Representative larvae from the "east Pacific barrier." A. Teleplanic veliger larva of an architectonicid species (×85) recovered at 3°36.7′S, 128°00.3′W, shown by arrow in Figure 2A. B. Details [illegible] same specimen rotated 90° counterclockwise showing umbilical teeth, callus at end of circumape[illegible] varix where it ends on inner lip and fine thread extending around edge of final whorl (×300). Other [illegible] of gastropods encountered in the east Pacific barrier include but are not limited to Bursidae, [illegible] Coralliophilidae, Thaiididae, and Cypraeidae. C. Enlargement of the setae (×300) of the ch[illegible] specimen shown in Figure D. D. Chaetosphaera larva (×100) belonging to a species of spioni[illegible] position assumed when disturbed or preserved—recovered from sample taken west of the [illegible] at 7°06.9′S, 142°59.9′W shown by arrow. E. Enlargement of the setae (×300) of the chaetosphaera specimen shown in Figure F at right. F. Chaetosphaera larva (×65), possibly the same species as in C and D above, recovered from a sample taken in the east tropical Pacific at 6°04′N, 91°57′W shown by arrow. The larvae appear most closely to resemble type VI described by Bhaud (1986) from New Caledonia. Scales at lower right denote 100 μm.

One possible hypothesis for the lack of successful dispersal of Panamic species across the east tropical Pacific is the absence of an extensive coastline that westward drifting colonizers may encounter. Only scattered islands provide the possibility for larval settlement. Nonetheless, east Pacific islands for which this argument might also apply apparently are colonized by eastwardly dispersing larvae. There are, of course, other determinates for successful immigration in either direction, for example the access to habitats in which colonizing species can survive and reproduce.

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Temperature Sensitivity of Molluscan and Arthropod Hemocyanins

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Abstract. The temperature sensitivity of hemocyanin-oxygen affinity and cooperativity was measured at 5, 15, 25, and 35°C in a variety of marine molluscs and arthropods from different thermal environments. These environments included a subtidal habitat in which the temperature is generally less than 15°C and the diurnal temperature variation is small, and an intertidal habitat in which the temperature varies more than 30°C. The temperature sensitivity of P_{50} showed considerable variation ($\Delta H = 0$ to $\Delta H = -67$ kJ/mol) depending on species and experimental temperatures. Sensitivity generally decreased as temperature increased. In several species temperature sensitivity was either absent or greatly reduced above 15°C. The horseshoe crab *Limulus polyphemus* showed a minimum temperature sensitivity between 15 and 25°C but higher sensitivity above and below this range. The hypothesis that a greater interaction between hemocyanin molecules and calcium ions at high temperatures offsets the temperature effect, resulting in a pigment less sensitive to temperature, was supported in an experiment where calcium ions were removed. Finally, delipidation of hemocyanin resulted in little or no change in oxygen affinity at all temperatures investigated.

Introduction

Long ago it was shown that O_2 affinity of the hemocyanins (Hcs), like almost all other O_2 carriers, decreases as temperature rises (Redfield, 1934). The range of temperature sensitivity is quite large, however, and exceptions to the general rule are known. While Miller and Van Holde (1981) retracted an earlier report of reversed temperature sensitivity of thalassinid Hc at low temperature, Morris *et al.* (1985) and Sanders and Childress (1985) both reported decreases in the O_2 affinity of other crustacean Hcs at low temperature. As is also true of most other O_2 carriers, temperature sensitivity of HcO_2 affinity varies within a species, often becoming smaller as temperature rises (Mauro and Mangum, 1982a, b; Bridges, 1986). In at least two species of terrestrial crustaceans, however, the temperature sensitivity of HcO_2 affinity is smallest in the temperature range in which the animals live (Morris and Bridges, 1985, 1986). Thus, the temperature sensitivity of HcO_2 affinity would appear to be variable and, at least occasionally, adaptive.

Oddly, few investigators have reported the effects of temperature on the cooperativity of HcO_2 binding. Mauro and Mangum (1982a, b) found the expected increase with temperature as molecular structure becomes less closed; the same trend appears to be present in the two curves shown by Angersbach and Decker (1978). However, no clear trend can be discerned in data shown by Jokumsen *et al.* (1981) and Bridges (1986).

In none of these investigations have temperatures above 25°C been examined, and yet many Hc-containing species do, in fact, experience such high temperatures. Moreover, all of the information summarized above pertains to crustacean Hcs. The few data available for chelicerate and molluscan Hcs (reviewed by Redfield, 1934; Mangum, 1980) suggest the same trends of an inverse relationship between temperature and its effects on HcO_2 affinity, and a direct relationship between temperature and its effects on cooperativity.

In an attempt to characterize the general thermal behavior of the hemocyanins, at least as a starting point, we have investigated the effects of temperatures from 5

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to 35°C on HcO_2 affinity and cooperativity, using both arthropod and molluscan Hcs and choosing species representing two quite distinct thermal environments. One group, designated cold water species, consists of subtidal crabs and an abalone collected from coastal waters of southern California and Puget Sound where seasonal temperature changes are small. The other group, designated the eurythermal species, consists of intertidal (at least transiently) and semi-terrestrial arthropods and molluscs, which originated from a variety of localities where the temperature is generally higher and quite variable.

We have also tested further the hypothesis that a lipid moiety of hemocyanin is related to the temperature dependence of HcO_2 binding (Mangum *et al.*, 1987), since it has been suggested previously that the lipid moiety of Hc influences O_2 binding in a way that might explain the seasonal change reported by several investigators (Zatta, 1981; Mauro and Mangum, 1982a). Finally, since ionic activity also increases with temperature, we have examined the role of the allosteric modulator Ca^{+2} in the temperature dependence of HcO_2 binding.

Materials and Methods

Experimental animals

The cold water species, represented by the crabs *Cancer anthonyi* (Rathbun), *C. gracilis* (Dana), and *Lopholithodes foraminatus* (Stimpson), and the pink abalone *Haliotis corrugata* (Gray), were held in running seawater at approximately 15°C. Hemolymph from another cold water crab, *C magister* (Dana), was kindly furnished by D. D. Jorgensen. Individuals of the most terrestrial (and therefore, eurythermal) crustacean studied, *Eurytium albidigitum* (Rathbun), were obtained from the northern Gulf of California at Laguna Percebu, 16 km south of San Felipe, Baja California, Mexico, and transported to San Diego, where they were held at 23°C. This species experiences temperatures ranging from about 15 to 36°C (Burnett and McMahon, 1987). Hemolymph was sampled from the intertidal chiton *Stenoplax conspicua* (Carpenter) *in situ* at Bird Rock, San Diego, where the air temperature was about 23°C and the water temperature 15°C. This eurythermal species experiences temperatures ranging from about 11 to 25°C. The chelicerate *Limulus polyphemus* (Linnaeus) was collected from the seaside coast of Virginia and held in recirculating seawater at 18–20°C. This species, which becomes intertidal only during its spring migrations into the estuary, experiences air temperatures ranging up to 30°C and water temperatures below 5°C.

O_2 equilibria

Hemolymph was sampled from the animals held under the conditions described above and oxygen equilibrium curves were determined at 5, 15, 25, and 35°C (except where noted) using techniques described below.

O_2 equilibria of *L. polyphemus* Hc were obtained by the cell respiration method (Mangum and Lykkeboe, 1979). All other data were collected tonometrically (Burnett, 1979; Burnett and Infantino, 1984). Temperature was controlled in all cases using thermostated water baths ± 0.1°C. The hemolymph samples from arthropods were allowed to clot and the clot disrupted using a glass homogenizer. All samples were centrifuged and 0.1 ml of the supernatant was added to 4.5 ml buffered saline. In two cases Hc concentration was low and required the addition of larger volumes of the supernatant (0.5 ml for *H. corrugata* Hc and 1 ml for *C. magister* Hc). The preparations were equilibrated to mixtures of N_2 (99.99% pure and <0.05 ppm O_2) and either air scrubbed of CO_2 and water, or O_2 (estimated 99.7% pure). Percent HcO_2 was estimated at 345 nm (Bausch & Lomb Spectronic 21 colorimeter).

The physiological salines used for *C. anthonyi*, *C. gracilis*, *C. magister*, and *L. foraminatus* Hcs were the same as that used earlier for *C. anthonyi* Hc (Burnett and Infantino, 1984). The saline used for *E. albidigitum* Hc was the same as that used for *Uca princeps* Hc (Burnett and Infantino, 1984), and the saline for *S. conspicua* Hc was that used for *Cryptochiton stelleri* Hc (Mangum and Burnett, 1986). The salines were buffered with either 0.05 mol/l HEPES, using HCl or NaOH to adjust pH, or 0.05 mol/l Tris maleate.

The data were described by regression lines (pH *versus* log P_{50}) and, if the slopes were homogeneous, differences in the Y intercepts assessed by analysis of covariance. In addition, the slopes of the regression lines describing log P_{50} as a function of pH were tested for differences from zero using a student's *t*-test. Temperature sensitivity of oxygen affinity was analyzed using van't Hoff plots where log P_{50} is plotted against 1/T (in degrees Kelvin) and the resulting slope is proportional to the heat of oxygenation, ΔH, *i.e.* slope = ΔH/gas constant. The data used for these plots were obtained from regression analysis of pH *versus* log P_{50} at different temperatures. This method of analysis allowed us to determine the effects of temperature on oxygen affinity at constant pH. In some cases positive values for ΔH resulted, which we attribute to data scatter especially in the cases of *C. gracilis* and *L. foraminatus*.

Sera were delipidated as described by Mangum *et al.* (1987). 0.02 g Triton X 100/g Hc [estimated by measuring the absorbance of hemolymph diluted with 10 mmol/l EDTA at pH 8.9 to eliminate light scattering and

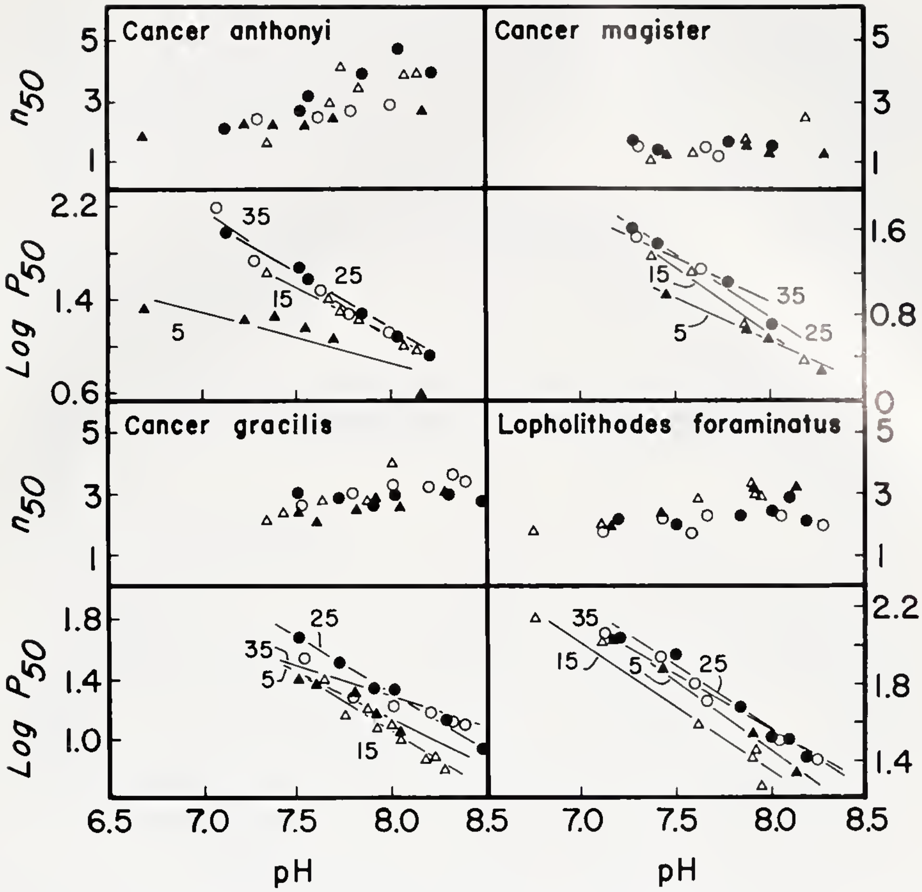

Figure 1. The effect of temperature on hemocyanin oxygen affinity (log P_{50}) and cooperativity (n_{50}) as a function of pH in four "cold" water crabs. *Cancer anthonyi, Cancer magister, Cancer gracilis,* and *Lopholithodes foraminatus.* P_{50} and n_{50} were determined at 5°C (▲), 15°C (△), 25°C (●), and 35°C (○).

using extinction coefficients reported by Nickerson and Van Holde (1971)] was combined with serum and stirred at room temperature for 1 h. The lipid-detergent complex was removed by adding 1 g Bio-Beads (SM-2 20-50 mesh. BioRad Co.)/g Hc and stirring for another h. The Bio-Beads were then removed by filtration through cotton.

Calcium was removed from samples of *C. anthonyi* hemolymph by dialysis overnight against two changes of 500 mmol/l NaCl and 1 mmol/l EGTA (ethyleneglycol-bis-N,N-tetra-acetic acid) in a ratio of 1 volume of sample:500 volumes of dialysis medium.

Results

In five of the eight species studied (the exceptions being *S. conspicua, L. foraminatus,* and *L. polyphemus*) temperature sensitivity of HcO_2 affinity is generally lowest at the highest temperatures (Figs. 1, 2, 3). In the van't Hoff plots (Fig. 3) this is represented by slopes which approach zero. In all species the Bohr coefficients (Δ log $P_{50}/\Delta pH$) differ significantly from zero ($P < .01$) throughout the temperature range.

In the five cold water species, temperature sensitivity of P_{50} is absent or, in *H. corrugata,* lowest between 25 and 35°C (Figs. 1, 2; Table I). This is most easily seen in the van't Hoff plots (Fig. 3). HcO_2 affinity in *C. gracilis* and *C. magister* also does not change significantly from 5 to 15°C at the physiological pH (7.8) (Table I). Surprisingly, a large positive value for ΔH was found in *L. foraminatus* between 5 and 15°C. However, if ΔH is calculated for the interval between 5 and 25°C, small negative values between −6.7 and −8.3 kJ/mol over the pH range result.

In contrast, HcO_2 affinity in the three eurythermal species changed significantly with temperature throughout the range examined with the exception of *E. albidigitum* at low pH (Fig. 2; Table I). The Hc of *E. albidigitum* also showed the trend of decreasing temperature sensitivity at

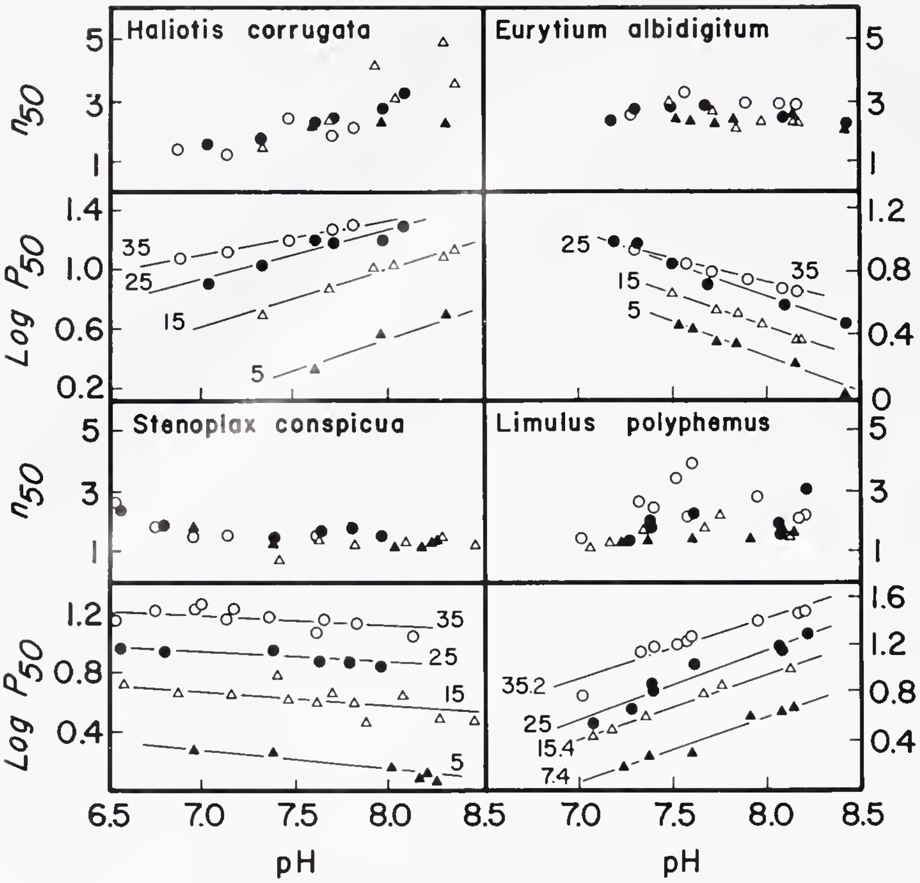

Figure 2. The effect of temperature on hemocyanin oxygen affinity (log P_{50}) and cooperativity (n_{50}) as a function of pH in the abalone *Haliotis corrugata*, the chiton *Stenoplax conspicua*, the xanthid crab *Eurytium albidigitum*, and the horseshoe crab *Limulus polyphemus*. P_{50} and n_{50} were determined at 5°C (▲), 15°C (△), 25°C (●), and 35°C (○), except as noted for *L. polyphemus*.

higher temperatures. In *L. polyphemus* minimal sensitivity was found in the 15 to 25°C range. Later it was found that this phenomenon is due to complete insensitivity between 20 and 25°C and that the ΔH value for 15–20°C is unexceptional (C. P. Mangum and J. Ricci, in prep.).

The values for cooperativity of the arthropod Hcs show some tendency to increase in the middle and upper ends of the pH range examined (Figs. 1, 2). In *C. anthonyi* and *H. corrugata* the pH dependence of cooperativity appears to increase with temperature; in the other species there is no clear trend. In general, however, cooperativity is influenced very little by either pH or temperature. Like other polyplacophoran Hcs (*e.g.*, Mangum and Burnett, 1986), *S. conspicua* Hc exhibits very little cooperativity, a feature that does not change with temperature (Fig. 2). Above 5°C, cooperativity of *H. corrugata* Hc decreases with decreasing pH (Fig. 2), a finding that agrees with Ainslie's (1980) report of a decrease in n_{50} with increasing P_{CO_2} of three other *Haliotis* Hcs. Otherwise temperature has no clear effect.

Dialyzing *C. anthonyi* Hc against 500 mmol/l NaCl and 1 mmol/l EGTA caused large decreases in O_2 affinity (*cf.* Figs. 1, 4). The present results indicate that Ca^{+2} has an effect on temperature sensitivity. The temperature sensitivity of the Hc dialyzed against a calcium-free saline and EGTA was slightly less than the controls between 5 and 25°C but much greater than the controls between 25 and 35°C (Fig. 5). Between 5 and 25°C the differences between ΔH were greatest at low pH and opposite to that predicted by our hypothesis (see Discussion).

At the two temperatures investigated, delipidation of *C. anthonyi* and *S. conspicua* Hcs caused no significant changes in O_2 affinity or its temperature sensitivity (Fig. 6; Table II). This result agrees with an earlier finding for *Callinectes sapidus* Hc (Mangum *et al.*, 1987). Delipidation of *E. albidigitum* Hc appears to have induced a small but significant decrease in O_2 affinity. We view this result with caution, however, in part because it is opposite to the change reported by Zatta (1981) and in part because at 35°C it occurs only at high pH.

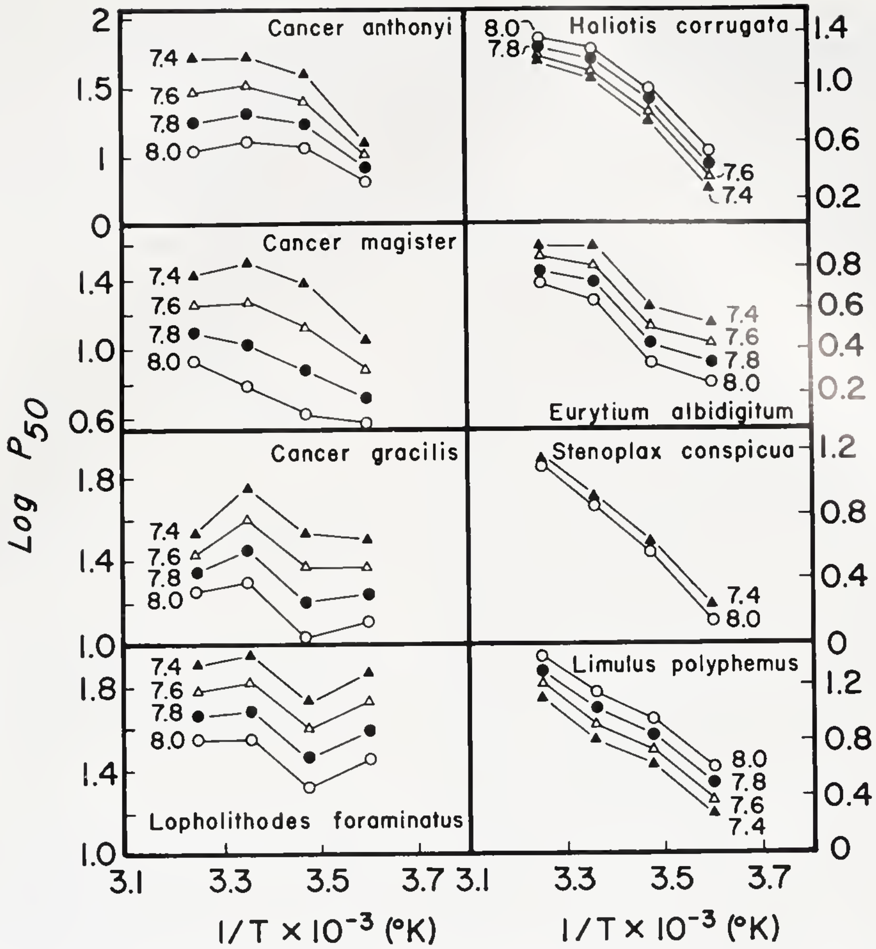

Figure 3. The effect of temperature on oxygen affinity at different pH expressed as van't Hoff plots: pH 7.4 (▲), pH 7.6 (△), pH 7.8 (●), pH 8.0 (○).

Discussion

In an evolutionary sense the O_2 binding properties of the crustacean Hcs have been considered rather conservative relative to those of some of the other O_2 carriers (Mangum, 1980). However, HcO_2 affinities are adaptable, both genetically and non-genetically. Of particular relevance here, higher O_2 affinities are found in species inhabiting warmer waters and lower O_2 affinities in species inhabiting colder waters (Redmond, 1968; Mangum, 1982; Mauro and Mangum, 1982b). The difference is adaptive because it offsets, in part, the intrinsic influence of temperature, *viz.* a decrease in HcO_2 affinity as temperature rises. The genetic adaptation enhances deoxygenation at the tissues in cold water species and oxygenation at the gill in warm water species. The latter may be especially important in species that encounter air (and must enhance the waterproofing of the cuticle, thus also increasing diffusion resistance of the gas exchanger) and in species that encounter hypoxic water. The adaptation is not perfect, however, and the available evidence indicates that the HcO_2 transport system does not play as large a role at low temperatures as it does at high temperatures (Mangum, 1980; Mauro and Mangum 1982b). The present results confirm the finding that the smaller role of the system at low temperature is due to a widespread increase in temperature dependence of O_2 binding.

A question arises as to why the various species have such different thermal sensitivities. ΔH values range from 0 to −70 kJ/mol in our sample and a [illegible] greater range is found in a larger sample (see Introduction). The answer may lie in the relationship between the relative magnitudes of temperature and pH dependence of the Hc, which appear to be inversely related. When the Bohr shift is normal and large, temperature dependence is small (*e.g.*, the intertidal species in the present sample) and vice versa (the cold water species in the present sample).

Table I

The effect of temperature on HcO_2 *oxygen affinity*

		log P_{50} vs. pH			
			Analysis of covariance		
		Bohr coeff.	Slope	Y-intcpt	ΔH (kJ/mol)
Cancer anthonyi	5	−0.49	NS	**	−49.2
	15	−0.88	*	NA	−13.3
	25	−1.02	NS	NS	10.0
	35	−1.11			
Cancer magister	5	−0.85	NS	NS	−25.1
	15	−1.25	NS	*	−24.7
	25	−1.18	NS	NS	−11.4
	35	−0.83			
Cancer gracilis	5	−0.65	NS	NS	5.7
	15	−0.82	NS	**	−41.4
	25	−0.75	**	NA	18.1
	35	−0.46			
Lopholithodes foraminatus	5	−0.70	NS	*	19.3
	15	−0.69	NS	NS	−36.9
	25	−0.67	NS	NS	4.0
	35	−0.60			
Haliotis corrugata	5	0.25	NS	**	−72.4
	15	0.35	NS	**	−46.2
	25	0.39	NS	**	−15.9
	35	0.44			
Eurytium albidigitum	5	−0.48	NS	**	−29.7
	15	−0.46	NS	**	−31.2
	25	−0.43	*	NA	−10.3
	35	−0.32			
Stenoplax conspicua	5	−0.15	NS	**	−64.4
	15	−0.12	NS	**	−47.3
	25	−0.07	NS	**	−43.2
	35	−0.09			
Limulus polyphemus	7.4	0.56	NS	**	−67.4
	15.4	0.53	NS	**	−33.0
	25	0.67	NS	**	−47.7
	35.2	0.05			

The analysis of covariance tests for differences in the slopes of regression lines fit to the data describing P_{50} as a function of pH for each Hc. If the slopes did not change with temperature, the analysis of covariance was used to test for differences in the Y-intercepts of the lines. ΔH was calculated from the value predicted by the regression analysis at pH 7.8. All slopes were different from zero according to a student's *t*-test. * = $.01 < P < .05$; ** = $P < .01$; NS = no significant difference at 0.05 level; NA = not applicable since slopes are significantly different.

When pH dependence is large, low temperature sensitivity minimizes the indirect effect of temperature due to the thermal sensitivity of hemolymph pH. The net effect of these interactions is to minimize the changes in temperature and oxygen affinity within a species. This is seen in Figure 7 where P_{50} is plotted as a function of temperature and pH is allowed to vary with temperature. While the absolute P_{50} may be dissimilar between species, the slope of the relationship between temperature and oxygen affinity within a species is relatively constant. Thus, oxygen affinity changes due to temperature are similar between species regardless of habitat. We emphasize that the relationship between hemolymph pH and temperature has not been measured in these six species but instead was predicted from the quantity $\Delta pH/\Delta °C = -0.016$ (Truchot, 1983); a number of exceptions to the rule are known (*e.g.*, Polites and Mangum, 1980). Figure 7 is intended only to illustrate the stabilizing potential of the interaction between pH and temperature sensitivities. This pattern appears to characterize at least some other Hcs with normal Bohr shifts (other studies). For example, the Hcs of the crabs *Uca princeps* and *Callinectes bellicosus* have moderate to large Bohr shifts (−0.71 and −1.32, respectively) and are relatively insen-

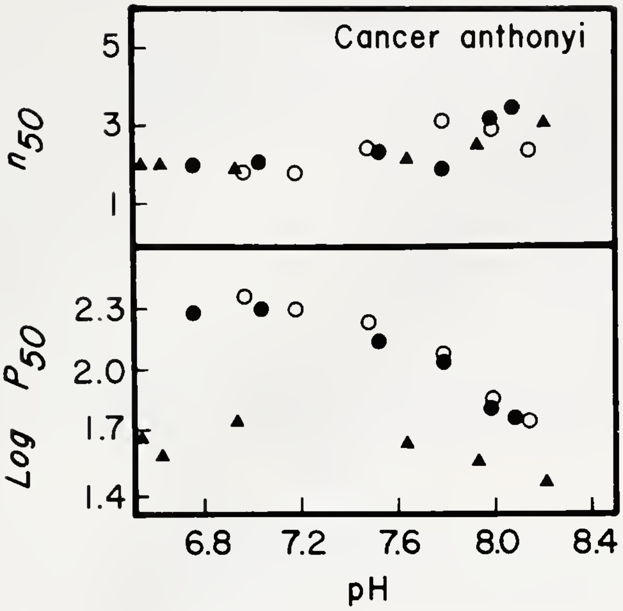

Figure 4. Oxygen affinity (log P_{50}) and cooperativity (n_{50}) of *Cancer anthonyi* hemocyanin in the absence of calcium ions as a function of pH and temperature; 5°C (▲), 25°C (●), and 35°C (○). Calcium ions were removed from the hemocyanin by dialyzing against 500 mmol/l NaCl and 10 mmol/l EGTA.

sitive to temperature (Burnett and Infantino, 1984). The freshwater crab *Holthuisana transversa* has a very small Bohr shift (<−0.2) and a pronounced temperature dependence (Morris *et al.*, 1988). However, there are also a number of Hcs with large normal Bohr shifts and a conventionally large temperature dependence as well (Jokumsen *et al.*, 1981; Mauro and Mangum, 1982a, b; Bridges *et al.*, 1983; Morris and Bridges, 1985, 1986); there must be additional selection pressures for Bohr shifts that may override the adaptive potential described here.

An indirect mechanism may be responsible for the decrease in temperature dependence at high temperatures which we have noted in some species. Andersson *et al.* (1982) showed that the number of available binding sites for the allosteric modulator Ca^{+2}, which raises O_2 affinity of Hcs with normal Bohr shifts, increases with temperature. One might also expect an increase in Ca^{+2} affinity of Hc at high temperature due to enhanced ionic activity and possibly other factors associated with changes in protein structure. We reasoned, therefore, that a greater interaction between calcium ions and Hc at higher temperature (*i.e.*, greater Ca^{+2} activity and more Ca^{+2}-Hc binding sites) would tend to increase oxygen affinity [provided that these Hcs respond to changes in free calcium ions as do those of other portunid crabs (Truchot, 1975; Mason *et al.*, 1983)] at a time when temperature acts to decrease it. The net result would be a depression of the temperature effect at higher temperature. This was indeed observed in our experiments where we removed calcium ions. We should mention that very small amounts of Mg^{+2}, which generally has a stronger effect than Ca^{+2} (Mason *et al.*, 1983), may have been present in our preparations since we chose not to use EDTA. It seems unlikely, however, that trace amounts would substitute exactly for the Ca^{+2} removed, and it seems more likely that the typical pattern of temperature dependence is a direct and intrinsic feature of calcium ion activity and/or protein structure. Furthermore, the presence of small amounts of Mg^{++} probably served to prevent the dissociation of the dodecamers into subunits (Ellerton *et al.*, 1970). Significant light scattering due to the presence of large subunits was observed in our dialyzed, deoxygenated samples. The light scattering was quantitatively similar (accounting for 5 to 19% of the absorbance in oxygenated samples) to control samples.

The response of *L. polyphemus* Hc to high temperature is also consistent with the above hypothesis. Greater calcium ion activity has an opposite effect on oxygen affinity causing an increase in P_{50} (Diefenbach and Mangum, 1983). Thus, at high temperature an increase in Hc calcium binding along with higher Ca^{+2} activity results in a large temperature-induced decrease in O_2 affinity. However, the responses of Hc of the hermit crab *Coenobita clypeatus* to temperature and calcium do not support the hypothesis. *C. clypeatus* has a small normal Bohr shift and temperature sensitivities which are smaller between 25 and 30°C but greater between 30 and 35°C (Morris and Bridges, 1986). While this pattern of temperature responses is similar to that found in *L. polyphemus*, changes in calcium ion binding to Hc and increases in calcium activity with temperature cannot be used to ex-

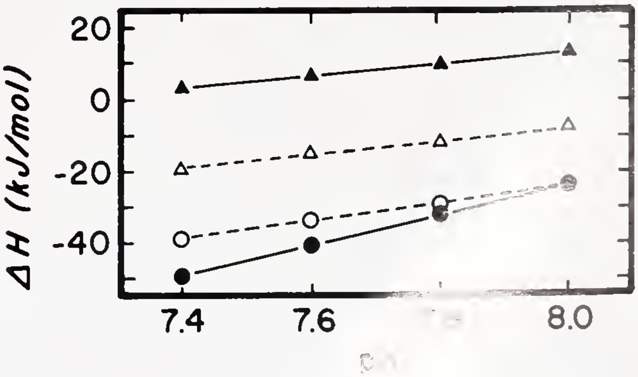

Figure 5. The heat of oxygenation of *Cancer anthonyi* hemocyanin between 5 and 25°C (circles) and 25 and 35°C (triangles) as a function of pH. Control values are represented by closed symbols while open symbols represent samples dialyzed against 500 mmol/l NaCl and 10 mmol/l EGTA.

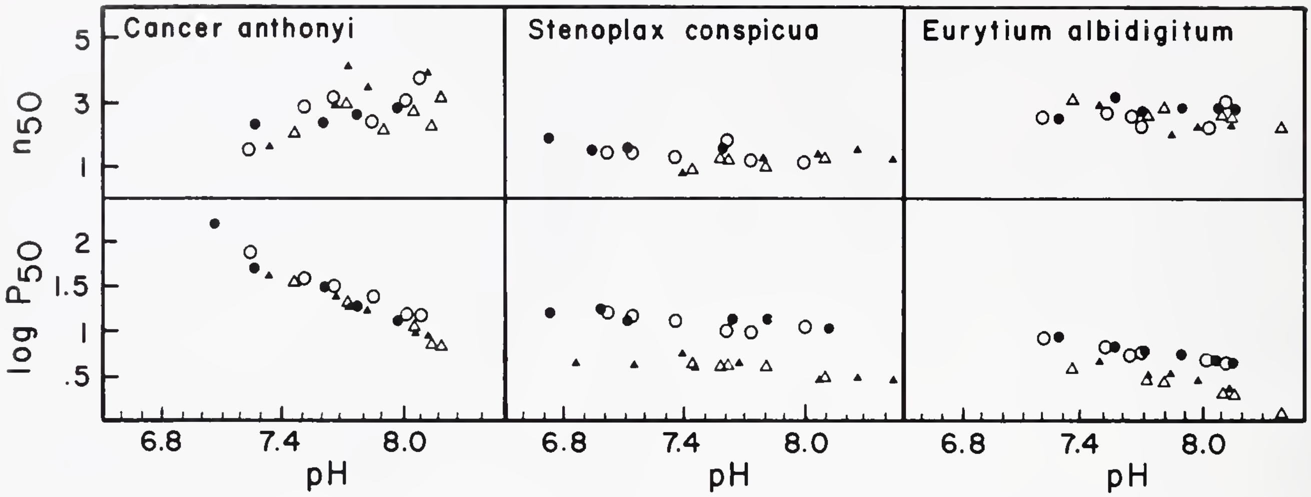

Figure 6. The effect of delipidation on *Cancer anthonyi*, *Stenoplax conspicua*, and *Eurytium albidigitum* hemocyanin oxygen affinity (log P_{50}) and cooperativity (n_{50}) as a function of pH and temperature; 15°C (triangles) and 35°C (circles). Values for delipidated hemocyanin are represented with open symbols; values for untreated hemocyanin are represented with closed symbols.

plain the increase in sensitivity at high temperature, since the oxygen affinity of *C. clypeatus* Hc is insensitive to changes in calcium ion concentration.

This hypothesis should be tested in future studies by measuring the temperature responses of oxygen affinity in Hcs from a variety of species that demonstrate a calcium sensitivity and from those that demonstrate no calcium sensitivity. If the presence of 3 to 17 low affinity calcium binding sites per subunit (Andersson *et al.*, 1982) is prevalent among the hemocyanins, it would in-

Table II

The effect of delipidation on the temperature dependence of HcO_2 binding

		n_{50} vs. pH				log P_{50} vs. pH			
				Analysis of covariance				Analysis of covariance	
	Temp.	Slope	Diff. from 0	Slope	Y-intercept	Bohr coeff.	Diff. from 0	Slope	Y-intercept
Cancer anthonyi									
control	15	2.74	*	NS	*	−0.88	**	NS	NS
delipidated	15	0.67	NS			−0.98	**		
control	35	0.63	NS	NS	NS	−1.11	**	NS	NS
delipidated	35	1.88	*			−0.80	**		
Eurytium albidigitum									
control	15	−0.86	NS	NS	*	−0.46	**	NS	**
delipidated	15	−0.70	*			−0.45	**		
control	35	0.15	NS	NS	NS	−0.32	**	NS	**
delipidated	35	0.16	NS			−0.29	**		
Stenoplax conspicua									
control	15	0.29	NS	NS	NS	−0.12	**	NS	NS
delipidated	15	0.17	NS			−0.18	**		
control	35	−0.88	NS	NS	NS	−0.09	**	NS	NS
delipidated	35	−0.20	NS			−0.20	**		

The slopes of regression lines fit to the data describing log P_{50} as a function of pH did not change with temperature or delipidation. The analysis of covariance was used to test for differences in Y-intercept values. * = $.01 < P < .05$; ** = $P < .01$; NS = no significant difference at 0.05 level.

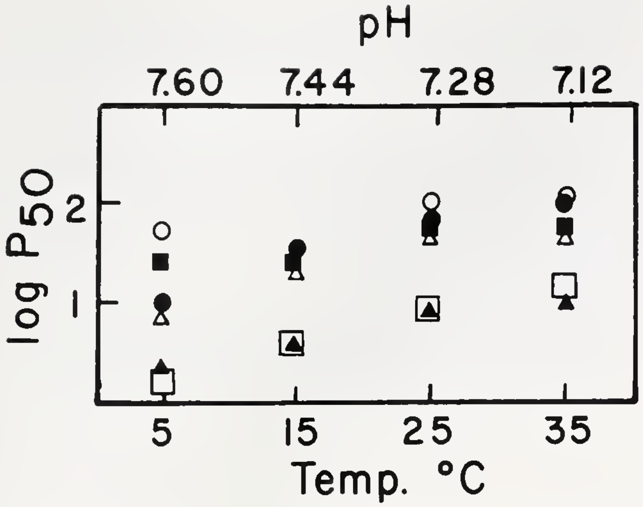

Figure 7. The relationship between temperature, where pH is assumed to vary according to ΔpH/Δ°C (Truchot, 1983), and the Bohr shift are plotted for *Stenoplax conspicua* (□), *Eurytium albidigitum* (△), *Cancer gracilis* (■), *Cancer magister* (▲), *Cancer anthonyi* (●), and *Lopholithodes foraminatus* (○).

dicate a new and physiologically important role for calcium in temperature responses.

Finally, in these species the lipid moiety of Hc apparently serves no purpose in stabilizing O_2 affinity over the thermal range investigated. These results are similar to those reported by Mangum *et al.* (1987), who showed that removal of serum lipids has no effect on oxygen binding in the crab *Callinectes sapidus.*

In summary, the effects of temperature on O_2 affinity of both arthropod and molluscan Hcs are highly variable. However, the most common pattern is greater sensitivity at low temperature and less sensitivity at high temperature. Furthermore, among the eight Hcs examined here, there was no sign of the reversed thermal sensitivity at low temperatures reported by Morris *et al.* (1985) and Sanders and Childress (1985), suggesting that it is not a particularly widespread adaptive mechanism.

Acknowledgments

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Control of Cnida Discharge: I. Evidence for Two Classes of Chemoreceptor

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Abstract. Appropriate chemical stimulation of cnidocytes along with mechanical stimulation is required to trigger discharge of cnidae. It has been generally assumed that such chemosensitization is mediated via specific chemoreceptors. Such chemoreceptors and their complementary ligands have never been identified. We now identify two groups of naturally occurring substances that chemosensitize cnida discharge in the feeding tentacles of the sea anemone, *Aiptasia pallida.* In addition, using a novel technique to quantify cnida discharge we demonstrate that these chemosensitizers act through at least two distinct classes of receptors. One class is broadly specific toward a variety of amino and imino acids and histamine ($K_{0.5}$ = 11–30 nM), but is competitively inhibited by antihistamines (K_i = 0.1–7.4 μM). A second class is specific for N-acetylated sugars ($K_{0.5}$ = 0.1–1.5 μM), but not affected by antihistamines. Presumably, these chemoreceptors detect specific substances from potential prey. Thus, cnidocytes are sensitized to discharge their cnidae in response to mechanical stimuli originating from the prey.

Introduction

Cnidocytes are secretory and sensory cells of cnidarians. They are located primarily on the tentacles of these animals. The cnidae, including the more commonly known nematocysts (Mariscal, 1984), develop within the cnidocytes and await appropriate stimuli to effect their discharge.

Cnidae are highly structured secretory products consisting of a small (5–200 μm), disulfide cross-linked (Blanquet and Lenhoff, 1966), collagen-like (Lenhoff *et al.,* 1957) capsule containing a hollow and eversible tubule continuous with the wall of the capsule (Cormier and Hessinger, 1980). They function primarily to capture prey (Ewer, 1947). The tubules of some cnidae, including certain nematocysts, evert rapidly (Holstein and Tardent, 1984) with enough force to penetrate prey and inject a lethal venom (Hessinger *et al.,* 1973; Tamkun and Hessinger, 1981). The tubules of other cnidae, including the spirocysts, are adhesive. They function to hold prey to the tentacles (Mariscal, 1984).

Cnida discharge involves the eversion of the tubule (Skaer and Picken, 1965) following proper stimulation of the cnidocyte. Parker and van Alstyne (1932) first demonstrated in the sea anenone, *Metridium senile,* and in the Portuguese Man-of-War, *Physalia physalis,* that *in situ* discharge of cnidae requires chemical stimulation and postulated the existence of chemoreceptors on cnidocytes. Subsequently, Pantin (1942) showed that discharge of *Anemonia sulcata* cnidae requires both chemical and tactile stimuli. Lubbock (1979) attempted to broadly identify the substances that sensitize cnidocytes to tactile triggering of cnida discharge by qualitatively testing 32 different high molecular weight, biological substances on the sea anemone, *Stichodactyla haddoni.*

In this report, using asexually cloned and cultured (Hessinger and Hessinger, 1981) sea anemones (*Aiptasia pallida*), we identify two groups of naturally occurring substances that chemosensitize cnidocytes for discharge. In addition, we show that the effects of these sensitizing substances are mediated by at least two distinct classes of chemoreceptor.

Materials and Methods

Maintenance of sea anemones

Sea anemones were cultured in natural seawater obtained from the Kerckoff Marine Laboratory of the Cali-

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fornia Institute of Technology in Corona del Mar, California. The animals used in these experiments were asexually cloned *Aiptasia pallida*, North Carolina strain (Hessinger and Hessinger, 1981). Clonemates of similar size and age were selected and individually reared in finger bowls containing approximately 250 ml natural seawater. Anemones were daily fed to repletion on freshly hatched *Artemia* nauplii (Hessinger and Hessinger, 1981) and maintained at 24 ± 1°C under a 12/12 h photoperiod using white fluorescent lights at an intensity of 5500 lux.

Experimental animals and test solutions

A group of animals was starved for 72 h prior to each experiment. These animals were kept under constant fluorescent light at 4500 lux during the last 48 h of the starvation period. This seemed to enhance uniformity of anemone behavior and cnidocyte responsiveness in experimental situations. Immediately before experimentation the animals were gently rinsed with fresh seawater to remove soluble wastes.

All test solutions were made in filtered (Whatman type 1), natural seawater adjusted to pH 7.65 with 1 *N* HCl or NaOH. Histamine, amino acids, N-acetylneuraminic acid, N-acetylglucosamine, bovine submaxillary mucin, and most other chemicals were purchased (Sigma, St. Louis, Missouri). Diphenhydramine and cimetidine were purchased from Parke-Davis (Morris Plains, New Jersey) and Smith, Kline and French (Philadelphia, Pennsylvania), respectively.

Qualitative and quantitative assays of discharge of cnidae

Assays of cnida discharge were based on the degree of adherence of sea anemone tentacles to suitable test objects. Such adherence is mediated by the everted tubules of cnidae such as the spirocysts and the microbasic p-mastigophore nematocysts, which, respectively, attach to and penetrate test objects. The extent to which tentacles adhere to test objects has been used to qualitatively detect the discharge of cnidae (Williams, 1968; Lubbock, 1979). We developed two methods for detecting and measuring discharge of cnidae by cnidocytes in anemone tentacles: a qualitative method for screening many naturally occurring biological substances for their ability to chemosensitize tentacle cnidocytes, and a sensitive quantitative method for studying dose-response relationships of chemosensitizers.

Qualitative screening assay. We qualitatively assessed the adherence of sea anemone tentacles to clean glass rods (1 mm diam) and to gelatin (30%, w/v) and to agarose (1%, w/v) pellets (5 mm diam and 3 mm length) fastened to a thin, steel wire wand. The tip of each glass rod was immersed in the test solution for about one min, and then air-dried. Pellets were soaked for two min either in filtered seawater (negative controls) or in test solutions. Glass rods and pellets on wire wands were presented by hand to the tip of one sea anemone tentacle for five seconds and then gently withdrawn. The response of cnidocytes on the tentacles to the combined chemical and tactile stimuli was observed and graded semi-quantitatively on the basis of strength of tentacle adhesion to the test object in relation to negative controls. Adherence was qualitatively rated as 0 (none), 1 (slight to moderate), and 2 (strong). Negative controls were rated as 0. For each substance tested, we computed a weighted average score by combining scores for pellets and glass rods by first multiplying the possible scores (0, 1, and 2) by the total number of tentacles tested giving that score (a, b, and c, respectively), then adding each of those values (0a + 1b + 2c), and dividing by the total number of tentacles tested (a + b + c). The substances tested were ranked according to the ability to chemosensitize the tentacles as determined by their weighted average scores. Clean test objects were used as negative controls and both bovine submaxillary and gastric mucin were used as positive controls, giving maximum responses.

We initially screened a wide variety of substances for the ability to sensitize sea anemone cnidocytes to tactile triggering of cnida discharge. The substances included more than 60 biological or biologically active compounds, including most of the 32 compounds originally tested by Lubbock (1979), within five major categories: (i) proteins and glycoproteins; (ii) amino compounds; (iii) monosaccharides; (iv) poly- and mucopolysaccharides; and (v) lipids. Different animals were individually tested for each compound. Concentrations of the tested substances were varied depending on the ability of these substances to adhere to the glass rods. The following concentrations were used: proteins and glycoproteins (1% w/v); amino compounds (1% w/v); N-acetylated sugars (0.1 *M*) and all other monosaccharides (1 *M*), except amygdalin (1%); agarose (0.5% w/v); dextran and dextran sulphate (60% w/v); glycogen (30% w/v); sodium polypectate and starch (15% w/v); heparin (42% w/v); chondroitin-6-sulphate (30% w/v); hyaluronic acid (1.9% w/v); all lipids (0.1% w/v), except lysolecithin, sphingomyelin, and phosphatidyl ethanolamine (5 mg/ml). All lipids were dissolved in ethanol and briefly air-dried on pellets and completely air-dried on glass rods.

Quantitative assay. We developed a more accurate and sensitive measurement of tentacle adherence by using small nylon beads attached to a force-transducer (strain guage). Clean nylon beads measuring 0.80 ± 0.01 mm diameter were coated with a thin layer (0.06 mm) of gelatin (30% w/v) and stored for no more than 24 h at 4°C until used. Experiments were performed at 24°C by exposing single anemones to 250 ml of test solution in a finger bowl. Animals were allowed to recover from the physical disruption of changing the medium for ten min before measurements were taken. Each probe was used on four separate tentacles and each anemone was used for a maximum of 20 measurements. Measurements taken in the absence of sensitizers were subtracted from measurements taken in the presence of sensitizer to give corrected values for the effect of the chemosensitizer alone. Measurements of adhesive force were made with the coated beads attached to the strain gauge (Grass model FT-03) via a narrow steel shaft. To maximize sensitivity the resistance springs were removed from the strain gauge. A sensitivity of 1 mg with 5% variation was achieved. Adhesive force measurements are expressed in hybrid units of milligram-force (mgf), rather than dynes (or newtons), since there is negligible acceleration, thereby making contributions from Newton's second law insignificant (Miller, 1959). Calibrations were obtained with weight standards and data were collected using a chart recorder. Linear regression analyses of data to determine such dose-response parameters as the maximum response (E_{max}), the concentration of agonist that produces a half-maximum response ($K_{0.5}$), and the molar disassociation constant of a competitive inhibitor (K_i), were performed using the GRAFPAC graphics computer program (Dorgan and Hessinger, 1984).

Results

Qualitative screening

We qualitatively screened more than 60 different biochemicals for the ability to chemosensitize tentacle cnidocytes to tactile triggering of discharge. Substances tested included a wide variety of proteins and glycoproteins, amino compounds, saccharides, poly- and mucopolysaccharides, and lipids (see Materials and Methods). Three types of test objects were used to present simultaneously chemical and mechanical stimuli to the anemone tentacles: gelatin pellets, agarose pellets, and glass rods. Since the results of using gelatin and agarose pellets to test various substances were quite similar, we have combined these data in Tables I through IV. The data obtained by using glass rods were somewhat different and, therefore, have been presented separately on the same tables. Weighted averages from all three types of test objects are also presented and used to rank the abilities of the substances tested to chemosensitize cnidocytes for discharge.

Proteins. The cnidocyte responses to various proteins, glycoproteins, and mucins (Table I) were either moderate (0.8–1.2) or strong (1.7–2.0). All of the tested glycoproteins and mucins produced maximum responses of 2.0, as did one non-glycosylated protein, namely poly-L-lysine. Weaker responses were elicited only by non-glycosylated proteins.

Amino compounds. Responses to amino compounds fell within three categories (Table II). No significant responses were elicited by any of the three tested antihistamines or by reduced glutathione. Strong responses (1.7) occurred in response to leucine, proline, glutamine, and histamine, while most other tested amino compounds, including glycine and alanine, elicited moderate responses (0.7–1.0).

Mono-, poly-, and mucopolysaccharides. Responses to various monosaccharides and mucopolysaccharides also varied (Table III). The tested amino sugars evoked no cnidocyte response, while glucose, galactose, and inositol produced slight to moderate responses (0.4–1.0). On the other hand, N-acetylgalactosamine and N-acetylglucosamine produced moderately strong responses, while N-acetylneuraminic acid, amygdalin and fucose produced maximum responses.

Of the tested mucopolysaccharides, anemones responded moderately to chondroitin sulphate and strongly to hyaluronic acid. Heparin, which has N-sulphates on C-2 in place of N-acetyl groups, had no activity. We were unable to test chitin, a linear polymer of N-acetylglucosamine, due to its insolubility in water. None of the tested polysaccharides including agarose, dextran sulphate, glycogen, sodium polypectate, and starch, showed any sensitizing effect (data not shown).

Lipids. None of the lipids tested evoked more than a moderate response, if at all (Table IV).

From this survey it appears that two broad groups of low molecular weight substances are identifiable that chemosensitize tentacle cnidocytes: the N-acetylated sugars and a wide variety of simple amino compounds. A variety of high molecular weight substances containing N-acetylated sugars also sensitize cnidocytes. Of these, mucins, glycoproteins and certain acidic mucopolysaccharides are the most potent.

Quantitative analysis

Dose-response parameters. To accurately quantify the relative number of cnidae that discharged in response to selected chemosensitizing substances, we measured the force (mgf) required to separate the stimulated tentacle from a probe consisting of a gelatin-coated nylon bead attached to a force-transducer. We assume this adhesive force to be directly proportional to the number of cnidae that discharged and adhered to the coated nylon bead.

Dose-response curves of all tested sensitizers were biphasic, showing a sigmoidal region of sensitization at lower concentrations of sensitizer, a maximum response at higher concentrations, and a downward response at still higher concentrations. The dose-response curves for

Table I

Responses of cnidocytes of Aiptasia pallida *tentacles to various proteins, glycoproteins, and mucins*

Compound	Pellet			Glass rod			Weighted averages
	0	1	2	0	1	2	
None	19	1	—	10	—	—	0.03
α-casein	6	14	—	—	10	—	0.80
Cytochrome C (horse)	2	18	—	—	10	—	0.93
Pepsin (porcine)	—	20	—	—	10	—	1.00
Trypsin	—	20	—	—	10	—	1.00
Haemoglobin	4	16	—	—	—	10	1.20
Lysozyme (egg white)	—	—	20	—	10	—	1.67
Myoglobin (equine)	4	—	16	—	—	10	1.73
Ovalbumin (hen)*	—	—	20	—	—	10	2.00
Polylysine	—	—	20	—	—	10	2.00
α-globulin (bovine)*	—	—	20	—	—	10	2.00
Serum albumin*	—	—	20	—	—	10	2.00
Submaxillary mucin*	—	—	20	—	—	10	2.00
Gastric mucin*	—	—	20	—	—	10	2.00

Responses are graded as 0 (none), 1 (slight), or 2 (strong). A total of 30 tentacles are tested for each compound: ten times each on agarose pellets, gelatin pellets, and glass rods. Results obtained with agarose and gelatin pellets are combined. Weighted averages represent combined scores for all 30 trials (see Materials and Methods). * Indicates glycoproteins.

glycine and N-acetylneuraminic acid (NANA) are typical of two general types of chemosensitizer (Fig. 1), each showing a distinct maximum response or effect (E_{max}) as well as a concentration in the sensitization region producing a half-maximal response ($K_{0.5}$), but with the NANA response showing a distinctively narrow and reproducible peak. Similarly shaped curves were obtained for alanine, glutamine, proline, and histamine, on the one hand, and for N-acetylglucosamine, and bovine submaxillary mucin, on the other.

Data from the sensitization region of these dose-response curves can be linearized on double-reciprocal

Table II

Responses of cnidocytes of Aiptasia pallida *tentacles to various non-protein amino compounds*

Compound	Pellet			Glass rod			Weighted averages
	0	1	2	0	1	2	
Diphenhydramine	20	—	—	10	—	—	0
Tripelennamine	20	—	—	10	—	—	0
Cimetidine	20	—	—	10	—	—	0
Glutathione (reduced)	14	6	—	10	—	—	0.20
Aspartic acid	—	20	—	10	—	—	0.67
Glutamic acid	—	20	—	10	—	—	0.67
Valine	10	10	—	—	10	—	0.67
Lysine	14	—	6	—	10	—	0.73
Serine	6	14	—	—	10	—	0.80
Alanine	2	18	—	—	10	—	0.93
Glycine	—	20	—	—	10	—	1.00
Cysteine	—	20	—	—	10	--	1.00
Histidine	—	20	—	—	10	—	1.00
Hydroxyproline	—	20	—	—	10	—	1.00
Hydroxylysine	—	20	—	—	10	—	1.00
Leucine	—	—	20	—	10	—	1.67
Proline	—	—	20	—	10	—	1.67
Glutamine	—	—	20	—	10	—	1.67
Histamine	—	—	20	—	10	—	1.67

Table III

Responses of cnidocytes of Aiptasia pallida *tentacles to monosaccharides and to mucopolysaccharides*

Compound	Pellet 0	Pellet 1	Pellet 2	Glass rod 0	Glass rod 1	Glass rod 2	Weighted averages
A. Monosaccharides:							
Galactosamine	20	—	—	10	—	—	0
Glucosamine	20	—	—	10	—	—	0
Galactose	18	2	—	—	10	—	0.40
Glucose	8	12	—	7	3	—	0.50
Inositol	—	20	—	—	10	—	1.00
N-Acetylgalactosamine	4	—	16	3	—	7	1.53
N-Acetylglucosamine	2	—	18	—	—	10	1.87
N-Acetylneuraminic acid	—	—	20	—	—	10	2.00
Amygdalin	—	—	20	—	—	10	2.00
Fucose	—	—	20	—	—	10	2.00
B. Mucopolysaccharides:							
Heparin	20	—	—	10	—	—	0
Chondroitin-6-sulphate	—	20	—	—	10	—	1.00
Hyaluronic acid	—	—	20	—	—	10	2.00

plots using linear regression analyses to calculate $K_{0.5}$ and E_{max} values (Table V). The $K_{0.5}$ values from these curves fall into two numerical groups: values of 1–3 $\times 10^{-2}$ μM for the amino sensitizers; and values one to two orders of magnitude higher (0.1–1.5 μM) for the N-acetylated sugars and mucin. These differences in dose-responsiveness, along with the obvious chemical differences between these two groups of sensitizers, suggest that these two groups of sensitizers occupy different receptors.

Is there more than one type of cnidocyte chemoreceptor? To determine whether more than one type of cnidocyte chemoreceptor exists, we tested analogues of various sensitizers that might block the cnidocyte response to one type of sensitizer but not the other. Our procedure involved testing such substances for the ability to block discharge of cnidae in the presence of a known sensitizing agent (agonist), while not themselves sensitizing the cnidocytes. We found that certain antihistamines (diphenhydramine, tripelennamine, and cimetidine) fit these criteria, blocking sensitization by amino agonists, but not sensitization to N-acetylated sugars (Table V). In particular, low concentrations of diphenhydramine displaced the sigmoidal region of the dose-response curve to the right for each of the amino agonists. Thus, increasing levels of diphenhydramine progressively shifted the $K_{0.5}$ to higher agonist concentrations on dose-response curves and on double-reciprocal plots (Fig. 2), while not affecting E_{max} values. This inhibitory effect is reversible and characteristic of receptor systems blocked by competitive inhibitors and antagonists (Segel, 1976; Goldstein *et al.*, 1974).

Table IV

Responses of cnidocytes of Aiptasia pallida *tentacles to various lipids*

Compound	Pellet 0	Pellet 1	Pellet 2	Glass rod 0	Glass rod 1	Glass rod 2	Weighted averages
Phosphatidyl ethanolamine*	20	—	—	10	—	—	0
Squalene	16	4	—	10	—	—	0.13
Sphingomyelin	16	4	—	10	—	—	0.13
Lysophosphatidyl choline	14	6	—	—	10	—	0.53
Gangliosides (brain)	6	14	—	—	10	—	0.80
Phosphatidyl choline (egg yolk)	—	20	—	—	10	—	1.00
Dipalmitoyl phosphatidyl choline	6	8	6	—	10	—	1.00

* Also inactive were: cholesterol, cholesterol palmitate, testosterone, palmitic acid, oleic acid, and egg yolk lysolecithin.

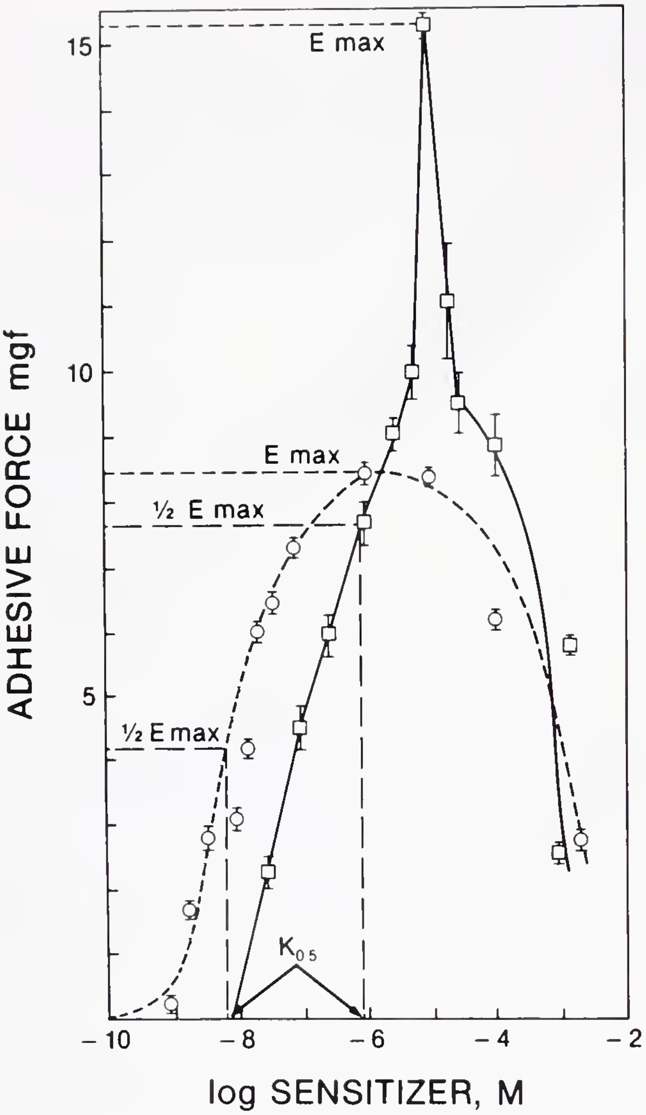

Figure 1. Dose-response curves of the cnidocyte response to glycine and N-acetylneuraminic acid (NANA). The dose-response curves for glycine (dashed line; mean n = 49, ranging from 31 to 72) and NANA (solid line; mean n = 90, ranging from 53 to 150) are typical of two types of chemosensitizing agents. Results are expressed as means of adhesive force (mgf) after correcting for adhesion with seawater alone, with vertical bars representing standard errors of means (95% confidence limits).

Dixon-type plots (Dixon, 1953) of the reciprocal of adhesive force *versus* inhibitor concentration yield straight lines for different concentrations of amino agonists (Fig. 3). These lines intersect at a common point that is indicative of competitive inhibition (Segel, 1976) and which gives the K_i for the antagonist.

Discussion

Results of our qualitative survey of more than 60 biochemicals for the ability to chemosensitize *A. pallida* cnidocytes confirm and extend the observations of Lubbock (1979) who used the large anemone *Stichodactyla haddoni.* Lubbock, using glass rods to present simultaneously the tactile stimulus and the chemical stimulus, showed that mucin and a few proteins—among 32 tested substances—allowed strong responses, while polysaccharides and lipids were virtually inactive. In the present survey we found that mucins, and specifically glycoproteins and a mucopolysaccharide, chemosensitize cnidocytes. In addition, however, we found that a wide variety of amino compounds and certain sugars strongly sensitize cnidocytes in the tentacles of *A. pallida.* Furthermore, *A. pallida* responded much more strongly to hyaluronic acid and polylysine and much less to α-casein than did *S. haddoni.*

The present study demonstrates that cnidocyte chemosensitization occurs either with the sensitizers free in solution (Table V) or adsorbed to a biologically inert gel (Tables I–IV). Furthermore, we have identified two groups of naturally occurring, low molecular weight substances that sensitize cnidocytes in the tentacles of the sea anemone, *A. pallida:* a variety of amino compounds (Table II) and three N-acetylated sugars plus amygdalin and fucose (Table III). The three N-acetylated sugars, all of which have an N-acetyl group on a hexose ring, are common constituents of glycoproteins, mucins, and mucopolysaccharides. Amygdalin, on the other hand, is a glycoside having a malenonitrile group attached to the C-1 of a hexose. Under certain conditions (*e.g.*, acid hydrolysis or alkaline peroxide attack) nitriles are converted to amides. In the case of amygdalin, the amide would be placed very close to C-2 of the hexose forming a close structural analogue to the N-acetyl hexoses. Fucose, while structurally dissimilar to N-acetylated sugars, is also a common constituent of mucins and glycoproteins.

While another group of substances, the antihistamines, do not sensitize cnidocytes (Table II), they do exert effects on the tentacle cnidocytes that are characteristic of receptor systems blocked by competitive inhibitors and antagonists (Segal, 1976; Goldstein *et al.*, 1974). They (i) displace dose-response curves of the amino agonists to the right, (ii) increase the $K_{0.5}$ values of amino agonists on double-reciprocal plots while not affecting E_{max} values (Fig. 2), and (iii) produce Dixon plots that yield straight lines having a common point of intersection (Fig. 3), which gives the K_i for the antagonist (Table V) and represents the dissociation constant of the receptor-inhibitor complex (Segel, 1976; Dixon, 1953). Therefore, we conclude that the antihistamine, diphenhydramine, acts as an antagonist at the amino receptor.

Due to the similarity of K_i values determined for diphenhydramine in the presence of proline, glutamine, histamine and, possibly, alanine (Table V), it seems

Table V

Dose-response parameters of chemoreceptor-mediated cnida discharge in Aiptasia pallida *tentacles*

	E_{max} (mgf)	$K_{0.5}$ (μM)	K_i (μM)
Glycine	7.95 ± 0.53	$1.09 \times 10^{-2} \pm 0.08$	0.129 ± 0.049
Alanine	8.51 ± 0.71	$3.00 \times 10^{-2} \pm 0.31$	7.35 ± 2.23
Proline	8.62 ± 0.61	$2.74 \times 10^{-2} \pm 0.22$	3.06 ± 0.58
Glutamine	10.07 ± 0.89	$2.40 \times 10^{-2} \pm 0.21$	3.06 ± 0.45
Histamine	14.22 ± 0.44	$2.04 \times 10^{-2} \pm 0.07$	2.42 ± 0.75
N-Acetylglucosamine	9.75 ± 0.48	0.115 ± 0.008	no effect
N-Acetylneuraminic acid	14.36 ± 1.51	1.46 ± 0.25	no effect
Submaxillary mucin	13.83 ± 1.29	1.55 ± 0.18	no effect

E_{max} is the adhesive force (mgf) required to separate an adhering tentacle from the probe tip at maximum sensitization. The $K_{0.5}$ represents the molar concentration of substance that half-maximally sensitizes the tentacle cnidocytes. Values of E_{max} and $K_{0.5}$ are determined from least-square double-reciprocal plots of the sensitization region of individual dose-response curves. K_i represents the molar dissociation constant of the receptor-inhibitor complex for a competitive inhibitor (*i.e.*, diphenhydramine) and is obtained as the x-intercept from linear plots of the apparent $K_{0.5}$ as a function of inhibitor concentration (Segal, 1976). The values of all parameters are expressed as means ± standard deviations.

likely that these amino agonists occupy the same or similar chemoreceptors, whereas the K_i value for glycine is significantly different. The antihistamines, however, have no effect on the cnidocyte response to the N-acetylated sugars or to bovine submaxillary mucin (Table V).

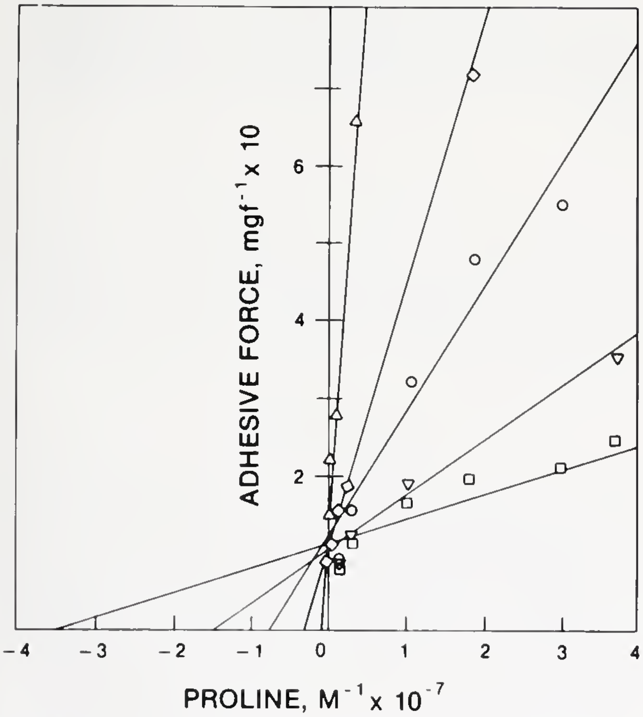

Figure 2. Double-reciprocal plots of proline-mediated sensitization in the presence of four different concentrations of diphenhydramine. The double-reciprocal plot of the sensitizing half of the biphasic dose-response curve is linear and yields $K_{0.5}$ and E_{max} values from x- and y-intercepts, respectively. Increasing concentrations of diphenhydramine cause an increase in the apparent $K_{0.5}$ value while not changing the value of E_{max}. Molar concentrations of diphenhydramine used were: no diphenhydramine (□); 5×10^{-7} (▽); 5×10^{-6} (○); 1.65×10^{-5} (◇); and 3.30×10^{-5} (△). Each data point is the mean of 60 or more tentacles with the standard errors being less than 0.002 mgf^{-1}.

We conclude that the cnidocyte sensitizing effect is mediated by surface chemoreceptors since the dose-response is saturable, and since the effect is reversible, specific, and can be competitively inhibited. We also conclude that there are at least two classes of cnidocyte chemoreceptors: (i) one class, with general specificity for a variety of amino and imino acids and histamine (Table II), but competitively inhibited by antihistamines (Figs. 2, 3); and (ii) a second class, specific for N-acetylated sugars and for high molecular weight substances bearing terminal N-acetylated sugars (Tables I, III), such as mucin, glycoproteins, and certain mucopolysaccharides, but not affected by antihistamines.

The fact that the oligosaccharide branches of bovine submaxillary mucin terminate in NANA residues (Herp *et al.*, 1979) suggests that mucin binds to the same chemoreceptor as free NANA. Furthermore, the nearly identical $K_{0.5}$ values for mucin and free NANA (Table V) suggest that a monovalent interaction of mucin with the "sugar" receptor is sufficient to chemosensitize, as opposed to requiring multivalent interaction and/or a clustering of NANA receptors.

These findings are the first to demonstrate and identify chemoreceptors involved in controlling cnidae discharge. Apparent desensitization of the receptor-mediated response of the cnidocytes is observed at high concentrations of both amino and N-acetylated sugar sensitizers. We speculate that such desensitization or sensory adaptation is a consequence either of receptor modification and/or of receptor-mediated endocytosis (Watson and Hessinger, 1987).

We propose a role for these cnidocyte chemoreceptors in the feeding process of cnidarians. Feeding by cnidarians involves two coordinated behaviors, namely, prey

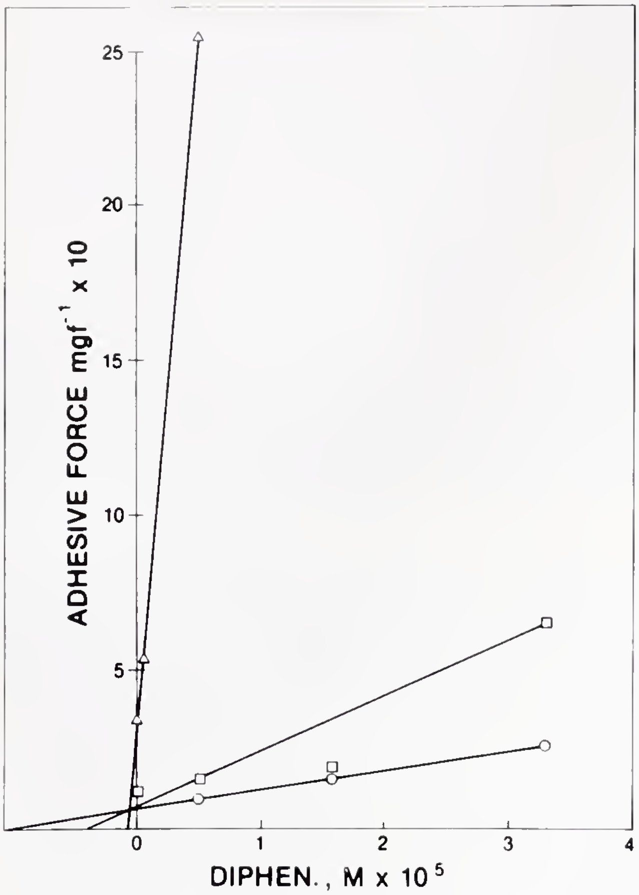

Figure 3. Dixon-type plots of proline-mediated sensitization in the presence of three different concentrations of proline. Molar concentrations of proline used were: (△) 2.7×10^{-8}; (□) 2.7×10^{-7}; and (○) 1×10^{-6}. Each data point is the mean of between 23 and 74 (average n = 40) tentacles.

capture and the feeding response. (i) Prey capture is effected by cnida discharge triggered by combined chemical and mechanical stimuli originating from the prey. Surface mucins on some prey (Daniel, 1981; Downing *et al.*, 1981) and the chitin exoskeletons of others, both of which either contain (Herp *et al.*, 1979) or are composed of (Austin *et al.*, 1981) N-acetylated sugars, may bind to one class of chemoreceptor and sensitize cnidocytes by lowering the threshold for discharging to mechanical stimulation. Upon discharge of the cnidae, the penetrant nematocysts puncture prey and inject nematocyst toxins that initially stimulate prey motor activity of the prey (Hessinger *et al.*, 1973), thereby increasing mechanical stimulation of cnidocytes. In addition, the puncture wounds caused by the penetrant nematocysts allow soluble substances, such as amino acids, to leak from the prey into the ambient medium. Some of those amino acids from the prey may sensitize additional cnidocytes via a second class of cnidocyte receptor causing additional cnidae to discharge. (ii) Following capture of the prey, the feeding response, involving a concerted movement of the tentacles to the mouth and the opening of the mouth, is also triggered by soluble substances leaking from punctured prey (Lenhoff and Heagy, 1977). In hydra the feeding response is induced by reduced glutathione (Loomis, 1955; Lenhoff, 1968; Cobb *et al.*, 1982), while in some sea anemones (Lenhoff and Heagy, 1977), including *A. pallida* (unpub. obs.), it is induced by some of the same amino compounds that sensitize cnidae to discharge. Thus, in many cnidarians, as in *A. pallida*, both prey capture and the feeding response are likely to be mediated by surface chemoreceptors that detect specific chemicals contained on and within suitable prey.

The present findings show that the control of cnida discharge can be studied at the molecular level. This cnida-cnidocyte system will be useful in unraveling both the mechanisms of sensory transduction in cnidocytes and the means by which chemical and mechanical stimuli are integrated to effect cnida discharge. The present approach to identifying and characterizing cnidocyte chemoreceptors involved in prey capture may also be useful in elucidating the mechanisms that control cnidocyte behavior as it relates to such phenomena as clonal aggression (*e.g.*, Francis, 1973) and tolerance of symbiotic anemonefish by certain sea anemones (*e.g.*, Schlichter, 1976).

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Identification and Origin of Hemoglobin in a Gymnophallid Metacercaria (Trematoda:Digenea), a Symbiote in the Marine Polychaete *Amphitrite ornata* (Annelida:Terebellidae)

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Abstract. Unencysted metacercariae belonging to the family Gymnophallidae (Trematoda:Digenea) were isolated from the metanephridial sacs of the common marine worm *Amphitrite ornata* (Polychaeta:Terebellidae). These metacercariae possess an intracellular red pigment that has characteristic absorption spectra for oxygenated, deoxygenated, and carbon monoxide derivatives of hemoglobin when exposed to oxygen, nitrogen, and carbon monoxide, respectively. The hemoglobin, found in all metacercaria tissues, has an oxygen half-saturation (P_{50}) value = 1.1 (S.D. = 0.3) mm Hg at 20°C and pH 7.0. The pigment shows cooperative oxygen binding with a Hill coefficient of 2.2 (S.D. = 0.2) and exhibits a significant Bohr effect between pH 6.8 and 7.4. The hemoglobin has a high molecular weight fraction ($\sim 2.5\text{–}3 \times 10^6$ daltons) and a 16,000 dalton MW fraction. HPLC ion exchange chromatography shows four distinct protein components. The host possesses both vascular extracellular hemoglobin (erythrocruorin) and coelomic cell hemoglobin. The metacercariae ingest host coelomic cell hemoglobin and probably vascular hemoglobin; however, metacercaria tissue hemoglobin is functionally and biochemically distinct from both host hemoglobins, suggesting an independent origin.

Introduction

Metacercariae in the family Gymnophallidae (Trematoda:Digenea) occur primarily in the extrapallial space of bivalve and gastropod molluscs (Cable, 1953; Stunkard and Uzmann, 1962). Exceptions recorded in the literature for three species in the genus *Gymnophallus* and one species in the genus *Parvatrema* include the use of polychaetous annelids as the second intermediate host (Oglesby, 1965; Margolis, 1971, 1973; Nikitina, 1976; Bartoli, 1981). Kyle and Noblet (1985) found unencysted gymnophallid metacercariae on the external body wall and tentacles of the marine polychaete *Amphitrite ornata* (Annelida:Terebellidae). Subsequently these larvae were found in the metanephridia of *A. ornata* (Burden and Noblet, 1987). These distome larvae range from 0.05 to 0.8 mm in diameter and from 0.1 to 1.5 mm in length. The metacercariae are a diffuse reddish-brown and many have bright red ceca. The identity of these larvae is unknown.

Although many trematodes are pink or red, only a few species have been shown to possess hemoglobin. The presence of hemoglobin in parasites was reviewed by Lee and Smith (1965). Since then only a few additional trematodes have been examined for hemoglobin (Lutz and Siddiqi, 1967; Cain, 1969a).

Based primarily on spectral evidence, Lee and Smith (1965) concluded that parasite hemoglobins were different from host hemoglobins. Lutz and Siddiqi (1967) determined by electrophoresis that the hemoglobin of *Fasciola gigantica* is distinct from its host hemoglobin. Cain (1969b) determined through radio-tracer experiments that *Fasciolopsis buski* synthesizes the globin moiety of its hemoglobin, but does not synthesize heme. Hemoglobins from two species of trematodes (*Fasciolopsis buski* and *Dicrocoelium dendriticum*) have been well-characterized biochemically (Cain, 1969a, c; Tuchschmid *et al.*, 1978; Di Iorio *et al.*, 1985; Smit *et al.*, 1986), but very little is known about hemoglobin function in any trema-

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tode parasite. Heme proteins, as identified by positive benzidine tests, occur in a few trematode larvae (Cain, 1969a), but the benzidine test does not distinguish between hemoglobins and other heme proteins or peroxidases.

The reddish-brown pigment of the gymnophallid metacercariae in *Amphitrite ornata* shows characteristic absorption spectra for hemoglobin, and the pigment exhibits reversible spectral changes upon alternate exposure to O_2 and N_2. Spectral examination of intact living animals showed hemoglobin to be present in every location of the metacercaria body.

Amphitrite ornata possesses both a vascular extracellular hemoglobin and a coelomic cell hemoglobin (Mangum *et al.*, 1975). Host vascular hemoglobin, sometimes termed erythrocruorin, is a high molecular weight molecule (MW = 3×10^6 daltons) found free in solution (hereafter referred to as HMW hemoglobin), while host coelomic cell hemoglobin is a monomer (MW = 16,000 daltons) (Weber *et al.*, 1977).

The bright red coloration of the ceca of metacercariae recently removed from the polychaete host suggests that the metacercariae may ingest hemoglobin from their host. Consequently, several possibilities were considered for the origin of hemoglobin in metacercaria body tissues. Metacercaria hemoglobin may represent one or both host hemoglobins or slightly modified versions of these pigments, or it may represent a unique pigment or pigments produced in the larval trematode from products derived from digestion of host hemoglobins and other substances (*i.e. de novo* synthesis). The present study was undertaken to characterize the physical and chemical properties of the hemoglobin found in tissues of these metacercariae and to compare metacercaria hemoglobin to host hemoglobins.

Materials and Methods

Specimens of *Amphitrite ornata* were collected from an intertidal mud flat at Garden City, South Carolina, and transported to Clemson University where they were placed in culture dishes in seawater. They were maintained at 16°C and the seawater was changed every one to two days. Metacercariae were removed from the metanephridia of *Amphitrite* and placed in fresh filtered (0.45 μm) seawater. They were used immediately for spectrophotometric analyses or were starved for 24 to 48 hours for use in biochemical analyses. Starvation ensured that host hemoglobins would not be present in the guts of the metacercariae. Following the starvation period, the animals were placed in a small amount of fresh filtered seawater and stored at −80°C.

Absorption spectra of host and metacercaria hemoglobins were obtained using a single beam microspectrophotometer as described by Colacino and Kraus (1984). Oxygen equilibrium measurements were made using a modification of a standard two wavelength technique (Rossi-Fanelli and Antonini, 1958). Animals were placed in 50 m*M* Tris buffered seawater between two teflon membranes (12.5 μm thick) in a specially designed gas slide within which gas tension and temperature were controlled (Colacino and Kraus, 1984).

Metacercariae used for histological sectioning were relaxed in three milliliters of seawater to which two to three small crystals of MS-222 (3-aminobenzoic acid ethyl ester) were added. Following relaxation, animals were fixed in 2.5% gluteraldehyde in Millonig's phosphate buffer (=960 mOsm). Postfixation was in 1% osmium tetroxide in the same buffer followed by alcohol dehydration and embedment in Polybed 812 resin. Whole animal cross sections (1 μm) stained with Methylene Blue and Azure B stain were photographed using a Zeiss Photomicroscope I. Ultrathin sections stained with lead citrate and uranyl acetate were examined on a Phillips 300 transmission electron microscope.

Metacercaria hemoglobin samples were prepared by sonicating thawed animals. Cell debris was removed by centrifugation at 16,000 × *g* for 2 minutes at 4°C. The resulting clear supernatant was placed on ice until used.

Amphitrite HMW hemoglobin was collected by first rinsing whole animals in 0.45 μm filtered seawater and blotting the animals on filter paper. The animals were then placed on parafilm and a gill filament was cut. The HMW vascular hemoglobin was pipetted into a centrifuge tube and placed on ice. The HMW hemoglobin was centrifuged at 16,000 × *g* for 2 min at 4°C to remove any cell debris or particulate contaminants and the clear red supernatant was diluted 1:1 with 0.1 *M* Tris buffered seawater, pH 7.4. Samples were placed on ice for immediate use, or were stored at −80°C for later analyses.

Amphitrite coelomocytes were collected by first cleaning the animals as described above, then making a small incision into the coelomic cavity. Coelomic fluid containing coelomocytes was pipetted into a centrifuge tube. The cells were washed two times with 0.05 *M* Tris buffered seawater, pH 7.4, then placed on ice. For chromatography, cells were lysed by freezing at −80°C. Cell debris was removed by centrifugation at 16,000 × *g* for 5 min at 4°C. The resulting clear red supernatant was placed on ice until used.

The pH of *Amphitrite* coelomic fluid was measured during collection of coelomic fluid. After the incision was made, a miniature pH electrode was placed into the coelomic fluid as it leaked from the opening. This method may introduce errors in the measured pH due to loss of carbon dioxide. After collection of the fluid, the sample was aerated and the pH remeasured. The pH was always more basic following aeration. Thus, errors in our

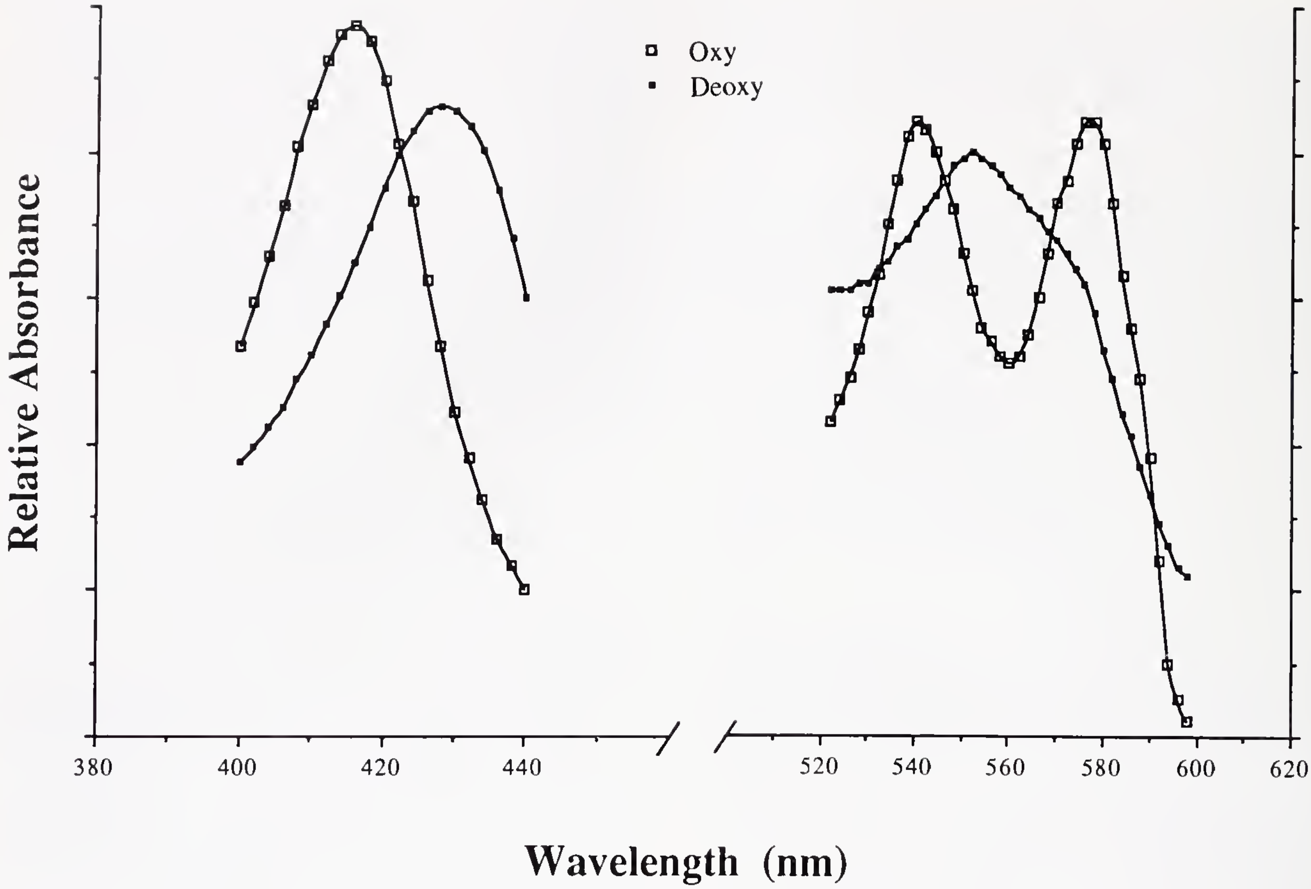

Figure 1. *In vivo* absorption spectra for metacercaria hemoglobin in oxygenated and deoxygenated states.

measured pH values due to rapid loss of carbon dioxide will be toward more basic pH's.

High Performance Liquid Chromatography (HPLC) gel filtration using a LDC Milton Roy CCM HPLC Automation system was performed on metacercaria hemoglobin, host HMW hemoglobin, and host coelomic cell hemoglobin samples to compare their approximate molecular sizes. Samples containing 10–50 μg/ml protein in 100 μl were eluted on a TSK-30 column, 7.5 × 300 mm (Bio-Rad), equilibrated with 0.05 *M* Tris, pH 7.4, at a flow rate of one ml/min and 500–1000 psi. Elution fractions as determined at 415 nm, an absorption maximum for heme proteins, were collected and stored at −80°C for later analysis. Metacercaria hemoglobin was also chromatographed on a 1 × 25 cm Sephacryl-400 column, equilibrated with the same buffer, to more closely determine the molecular weight of the high molecular weight fraction.

HPLC anion exchange chromatography was performed on metacercaria, *Amphitrite* HMW, and coelomic cell hemoglobins, and on the elution fractions from gel filtration experiments. Hemoglobin fractions were determined by detection at 415 nm. Separation was achieved using a TSK-DEAE-5-PW column, 7.5 × 75 mm (Bio-Rad), initially equilibrated with buffer A (0.01 *M* Tris, pH 9.0). A linear salt gradient was established from 0% to 50% buffer B over a 50 minute run time. Buffer B was 0.01 *M* Tris, pH 9.0, with 1.0 *M* sodium acetate. All reagents used were HPLC grade.

Incorporation of H^3-glycine into proteins by metacercariae was determined by incubating 250 animals for 24 hours at 16°C in 2.0 ml of 0.1 m*M* glycine (specific activity of 10 μCi/ml) in 0.05 *M* Tris buffered seawater, pH 7.4. Control animals were incubated in an identical buffer with unlabeled glycine for 24 hours at 16°C. All animals had been starved outside of the host for 12 hours prior to the start of the experiment. Following incubation, controls and experimentals were washed four times with 50 m*M* Tris buffered seawater, pH 7.4, at 0°C and then placed in 250 to 500 μl of 50 m*M* Tris buffer, pH 7.4. Samples stored at −80°C were thawed, sonicated for 15 seconds, centrifuged at 16,000 × *g* for 2 min to remove cell debris, and placed on ice. HPLC gel filtration was run on 100 μl volumes of control and experimental

Table I

Comparison of absorption maxima (nm) for metacercaria, Amphitrite ornata *and human hemoglobins*$^{\triangle}$

		Amphitrite ornata			
		Vascular	Coelomic	Metacercaria	Human
OXY	α	576.0 (0.8/8)	577.7 (0.5/5)	578.2 (0.6/13)	577.4 (0.5/8)
	β	541.1 (0.4/8)	540.9 (0.9/5)	541.3 (0.7/13)	542.5 (0.8/8)
	γ	418.4 (0.7/8)	416.1 (0.4/2)	415.7 (0.9/13)	415.4 (0.5/8)
DEOXY	$\alpha\beta$	554.7 (2.7/6)	555.5 (1.0/4)	559.9 (2.1/7)	555.4 (0.8/8)
	γ	430.2 (0.8/6)	431.5 (−/1)	428.5 (1.6/7)	430.8 (0.5/8)
CARBOXY	α	569**	570**	570.4 (2.9/2)	568.5*
	β	538**	538**	538.9 (0.4/2)	539*
	γ	421**	421**	420.6 (0.4/2)	420*

* Data are from Van Assendelft (1970).

** Data are from Chiancone *et al.* (1980).

$^{\triangle}$ The numbers in parentheses represent standard deviation/number of samples.

samples (see above for conditions). Sequential one-half ml fractions of the entire gel filtration eluent were collected and the amount of label in each determined using a Beckman LS7000 Liquid Scintillation Counter.

Results

In vivo spectrophotometry of metacercaria tissues showed characteristic hemoglobin spectra when the tissues were exposed to oxygen, nitrogen, and carbon monoxide. The *in vivo* absorption spectra for metacercaria hemoglobin are shown in Figure 1. The absorption maxima of metacercaria tissue hemoglobin and *Amphitrite ornata* HMW and coelomic cell hemoglobins are listed in Table I along with the maxima for human hemoglobin. Only minor variations in maxima are apparent between all four pigments. Absorption spectra of metacercaria gut contents gave absorption profiles that were not recognizable as typical for hemoglobin in oxy, deoxy, or met state (Van Assendelft, 1970).

Oxygen equilibrium curves for metacercaria hemoglobin *in vivo* are shown in Figure 2. The *in vivo* oxygen binding properties of metacercaria hemoglobin are summarized in Table II. The hemoglobin has a high affinity for oxygen and demonstrates cooperative oxygen binding. It exhibits a positive Bohr effect between pH 6.8 and 7.4 (−0.35). There is no significant Bohr effect between pH 7.4 and 7.8. The pH of *Amphitrite* coelomic fluid directly removed from the animal was found to range from 6.9 to 7.6, with a mean pH of 7.3 (SD = 0.2, n = 10). Following aeration of the coelomic fluid, the pH ranged from 7.3 to 7.8, with a mean pH of 7.5 (SD = 0.1, n = 10). The functional properties of metacercaria tissue hemoglobin are compared with *in vitro* oxygen binding characteristics of *Amphitrite* HMW and coelomic cell hemoglobins in Table III. The P_{50} (the O_2 partial pressure required to achieve half-saturation) of metacercaria hemoglobin *in vivo* is lower than either of the *Amphitrite* hemoglobins *in vitro*. Values for host vascular hemoglobin are similar to those reported by Mangum *et al.* (1975) and Chiancone *et al.* (1981). Our measurements of *in cellulo* P_{50} for *Amphitrite* coelomic cell hemoglobin gave slightly lower affinity (higher P_{50}) than the *in vitro* and *in vivo* (cell suspension) values listed in Table III from Mangum *et al.* (1975). Metacercaria tissue hemoglobin shows greater cooperativity than either hemoglobin from *Amphitrite*, and has a significant Bohr effect unlike the host hemoglobins.

Cross sections of the gut ceca of metacercariae showed the presence of *Amphitrite* coelomocytes. Other cells and debris were present which had the coloration and appear-

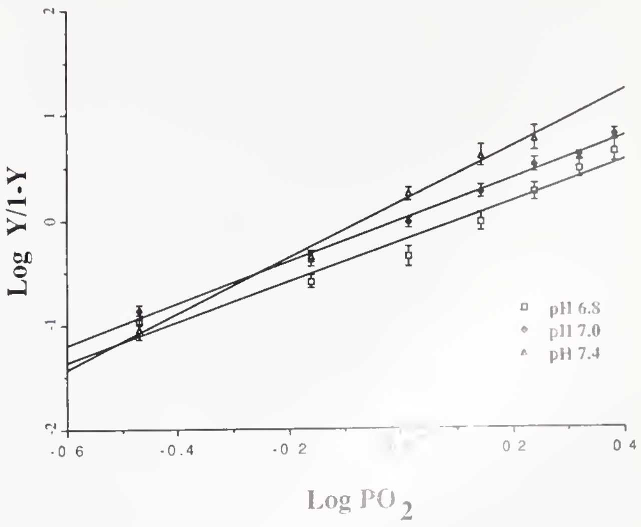

Figure 2. *In vivo* oxygen equilibrium curves for metacercaria hemoglobin. Vertical bars show standard error.

Table II

Oxygen binding characteristics of metacercaria hemoglobin

pH	P_{50} (mmHg)	Hill constant	n
6.8	1.32 ± 0.39*	2.25 ± 0.29	8
7.0	1.12 ± 0.29	2.22 ± 0.24	10
7.4	0.81 ± 0.22	3.06 ± 0.40	7
7.8	0.78 ± 0.23	2.39 ± 0.38	6

* S.D.

ance of the highly vascularized metanephridial tissue; however, transmission electron micrographs of gut ceca and gut lining cells revealed no evidence of material that resembled the characteristic annelid high molecular weight extracellular hemoglobins.

Figure 3 shows the elution profile of metacercaria hemoglobin from HPLC gel filtration which separates molecules on the basis of their size. Two fractions were eluted; fraction A eluted in the void volume of the column (MW > 500,000 daltons) and fraction B eluted at 16,000 daltons. Standard gel filtration on a Sephacryl S-400 column (Fig. 4) indicated that fraction A has a molecular weight of 2.5–3 × 10^6 daltons.

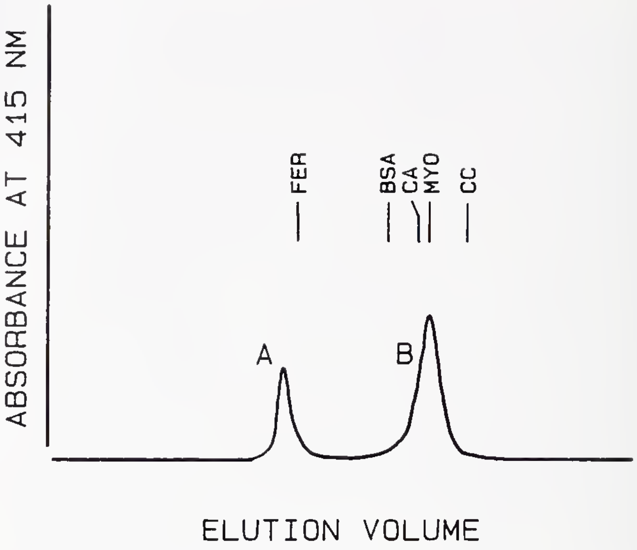

Figure 3. High performance liquid chromatography (HPLC) of metacercaria whole animal lysate. Key to standards: FER, ferritin; BSA, bovine serum albumin; CA, carbonic anhydrase; MYO, myoglobin; CC, cytochrome C.

HPLC ion exchange chromatograms of whole metacercaria lysate and gel filtration fractions of metacercaria hemoglobin are shown in Figure 5a–c. Ion exchange separation of whole metacercaria hemoglobin resulted in four distinct protein components (Fig. 5a). Metacercaria gel filtration fraction A (MW ~ 2.5–3 × 10^6 daltons) when chromatographed on the ion exchange column demonstrated one broad peak with a distinct shoulder, indicating the presence of two components that were incompletely separated (Fig. 5b). These components of fraction A probably correspond to protein components I and II of whole metacercaria hemoglobin (Fig. 5a). The ion exchange chromatogram of metacercaria gel filtration fraction B (MW ~ 16,000 daltons) is shown in Figure 5c. It demonstrated two peaks that probably correspond to components III and IV on the whole metacercaria hemoglobin chromatogram (Fig. 5a).

Table III

Comparison of oxygen binding characteristics of metacercaria and Amphitrite ornata *hemoglobins*

	P_{50} (mm Hg)	Hill constant	Bohr coefficient
A ornata			
coelomic cell			
in cellulo (pH 7.4)	3.35 (SD, 0.38)	1.20 (SD, 0.19)	—
cell suspension* (pH 6.8–7.8)	1.56–2.60	0.811–1.117	—
*in vitro** (pH 6.25–9.05)	2.72	0.91	NS
vascular* (pH 5.81–7.97)	10.00	1.286	NS
vascular** (pH 6.0–7.4)	6.3–10	≅1.5	—
Metacercaria (pH 6.8–7.4)	0.81–1.32	2.22–3.06	−0.35

* Data is from Mangum *et al.* (1975).
** Data is from Figure 3 of Chiancone *et al.* (1981).

Ion exchange chromatograms of *Amphitrite ornata* vascular HMW hemoglobin and coelomic cell hemoglobin are shown in Figure 5d–e. The ion exchange chromatograms of metacercaria whole hemoglobin (Fig. 5a) and the high molecular weight fraction of metacercaria hemoglobin (Fig. 5b) when compared with the elution profile of *Amphitrite* vascular hemoglobin (Fig. 5d) show no correspondence of any component. The ion exchange elution profile of *Amphitrite* coelomic cell hemoglobin (Fig. 5e) shows three protein components, all of which are distinct from the protein components that compose metacercaria whole hemoglobin (Fig. 5a) and the components of the 16,000 MW fraction of metacercaria hemoglobin (Fig. 5c).

Incorporation of H^3-glycine into proteins by these metacercariae was demonstrated. Figure 6 shows a gel filtration elution profile for metacercaria proteins compared to a histogram representing total CPM (background and label) recorded from sequential 1/2 ml elution fractions. The cross-striated portions of the absorbance profile indicate the location of hemoglobin fractions. These results demonstrate the presence of sig-

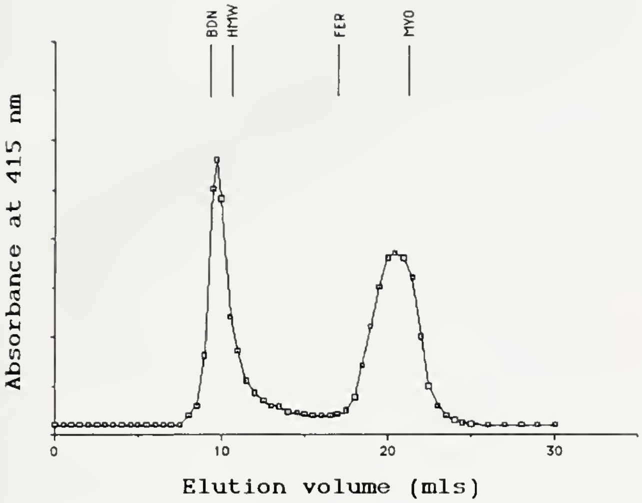

Figure 4. Sephacryl-400 chromatography of metacercaria whole animal lysate. Key to standards: BDN, blue dextran; HMW, *Amphitrite* HMW vascular hemoglobin; FER, ferritin; MYO, myoglobin.

nificant amounts of tritiated glycine in eluted fractions containing proteins which include hemoglobins.

Discussion

The absorption spectra of the pigment in metacercaria tissues identifies it as a heme protein. The pigment appears to be in all body cells but may also be located in intercellular spaces as in some entosymbiotic rhabdocoel flatworms (Jennings and Cannon, 1985). The metacercaria hemoglobin exhibited reversible spectral shifts when alternately exposed to oxygen and nitrogen, indicating a functional ability to bind oxygen. The absorption maxima for oxy, deoxy and carboxy forms of the hemoglobin closely resemble other functional hemoproteins, including both host hemoglobins (Weber *et al.*, 1977). Cain (1969a) demonstrated the presence of heme protein in several larval trematode stages by positive benzidine reaction. However, this is the first larval trematode shown to possess a hemoglobin capable of functional oxygen transport.

Hemoglobin-containing adult trematode, monogenean, nematode, and arthropod parasites are known from many vertebrate and invertebrate hosts (Lee and Smith, 1965; Thurston, 1970; Von Brand, 1973). Many of these parasites live in regions in the host that have low PO_2 such as the liver or the lumen of the gut. Some, like the nematode *Syngamus trachea* (Rose and Kaplan, 1972), live in high PO_2 environments. Hemoglobin-containing turbellarian worms of the families Pterastericolidae and Umagillidae have been identified living in the guts of their echinoderm hosts (Jennings and Cannon, 1985, 1987). As in other animals, hemoglobin in parasitic or endocommensal animals is presumed to function in facilitated diffusion, transport, or storage of oxygen in low PO_2 environments (Weber, 1980; Sharpe and Lee, 1981). Very little is known, however, about the actual function of hemoglobin in these symbiotes.

In general, hemoglobins in symbiotic helminths have high affinities for oxygen (Lee and Smith, 1965; Von Brand, 1973). Host hemoglobins, when present, have lower affinities than the symbiote hemoglobins. The high affinity symbiote hemoglobins may allow the symbiotes to extract oxygen from low PO_2 environments. In some parasitic nematodes such as *Ascaris* and *Strongylus*, the hemocoel hemoglobin affinities are so high that it is doubtful that these hemoglobins function in aerobic metabolism (Okazaki and Wittenberg, 1965). However, if a symbiote's hemoglobin unloads oxygen in the range of oxygen tensions encountered in the host tissues, it is likely to serve a respiratory function.

The gymnophallid metacercariae living inside the host metanephridia are separated physically from the host's coelomic cavity by the thin, highly vascularized walls of the metanephridia. During high tide with normal ventilation, transport of O_2 by HMW vascular hemoglobin in *Amphitrite* probably controls the PO_2 within both the metanephridia and the coelom. Mangum *et al.* (1975) determined that under these conditions, coelomic PO_2 would be close to the P_{50} of the HMW vascular hemoglobin (~10 mm Hg at physiological pH). At low external PO_2, as would be experienced during low tide, the vascular HMW hemoglobin fails to load oxygen at the gills (Mangum *et al.*, 1975). Thus, the primary supply for oxygen to the metanephridia and metacercariae is interrupted. At this time, the source of oxygen for the metanephridia and metacercariae is the coelomic fluid. The PO_2 inside the metanephridia probably declines below coelomic PO_2 (near coelomic cell hemoglobin P_{50} of 2.7 mm Hg) due to a combination of oxygen consumption by the metanephridial lining and consumption by the metacercariae which can occupy as much as 50% or more of the nephridial space. During these periods of hypoxia, the high affinity hemoglobin of the metacercariae ($P_{50} = 1.2$ mm Hg) may allow them to obtain oxygen at greater rates by facilitated diffusion. Under anoxic conditions, it may allow them to extend aerobic metabolism by acting as a short term store.

Amphitrite coelomocytes that are swept into the metanephridia through the nephrostome are observed in the gut of the larvae. Although the metanephridial lining is highly vascularized and at most only one cell layer separates the host vascular hemoglobin from the trematode larvae, we were unable to demonstrate ingestion of host HMW hemoglobin by metacercariae. However, if metacercariae are feeding on metanephridial tissue as is suggested by the appearance of similar material in their gut

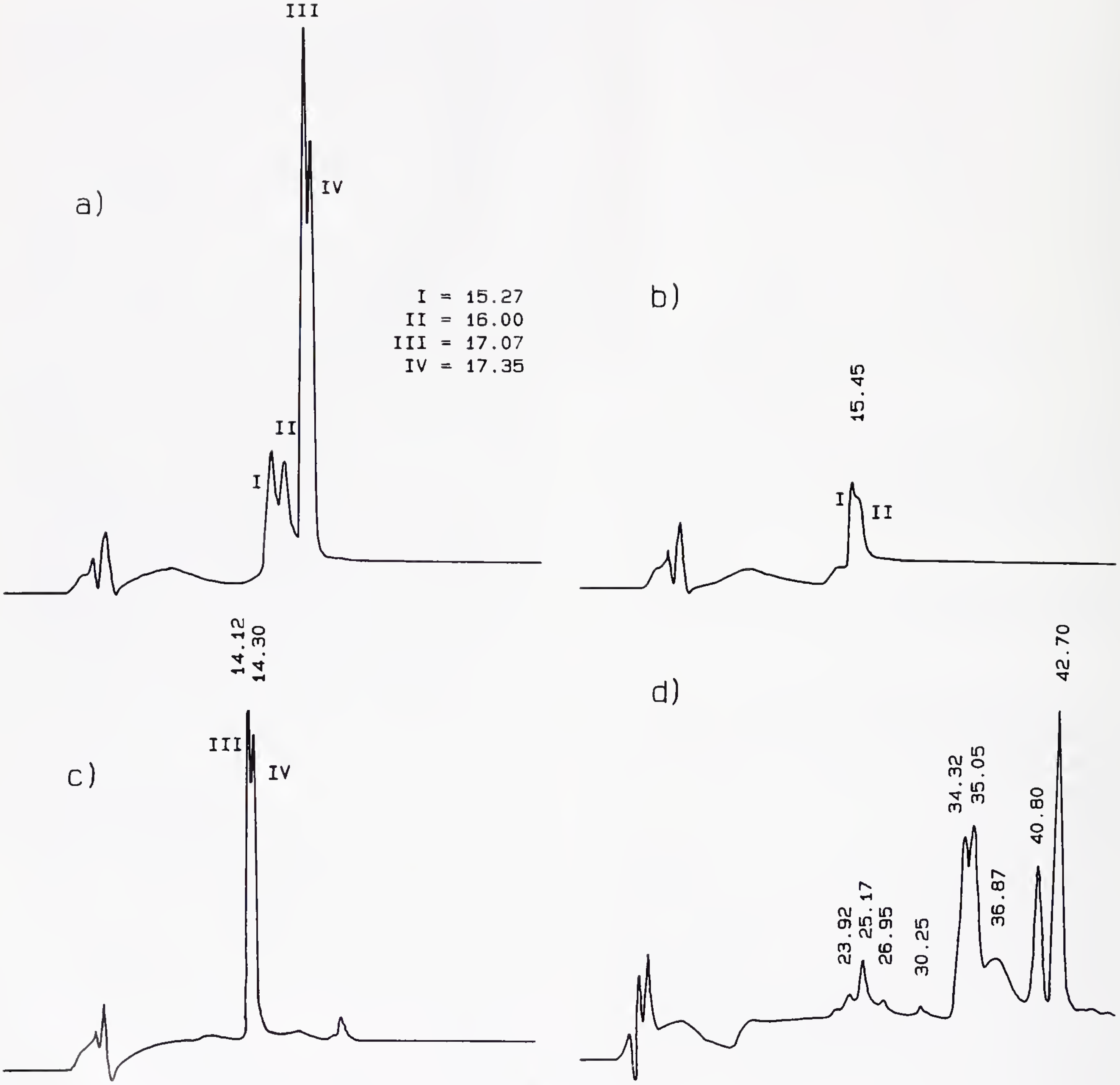

Figure 5. HPLC anion exchange chromatography of a) metacercaria whole animal lysate, b) metacercaria gel filtration fraction A*, c) metacercaria gel filtration fraction B*, d) *Amphitrite ornata* HMW vascular hemoglobin, and e) *Amphitrite ornata* coelomic cell hemoglobin. Chromatograms are not to scale. Absorbance monitored at 415 nm. Decimal numbers represent elution times.

* These samples were loaded onto the ion exchange column in gel filtration buffer which had a different ionic strength from the other samples which were at the ionic strength of seawater. Therefore, elution times will not compare.

ceca, then it is probable that ingestion of host vascular hemoglobin might also occur. Ingestion of host hemoglobin as a source of nutrition is a common occurrence among vertebrate parasites (Halton, 1967; Schmidt and Roberts, 1985) but until now has not been observed among parasites of invertebrates.

Halton (1974) showed that hemoglobin ingested by the monogenean *Diclidophora merlangi* is taken up directly into cecal cells by pinocytosis and then broken down. If gut lining cells of the metacercaria larvae took up intact *Amphitrite* hemoglobins by pinocytosis as does *Diclidophora*, it is possible that these hemoglobins may

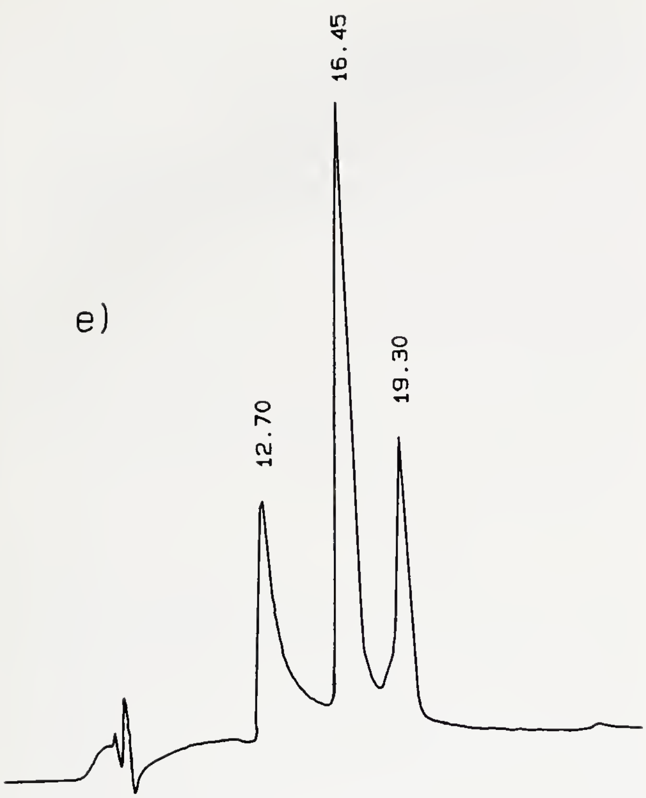

Figure 5. Continued.

not be completely degraded or not broken down at all, but rather retained and functionally altered.

The metacercariae possess a monomeric hemoglobin with an approximate molecular weight of 16,000 and two different globin forms. This corresponds to the results found for the trematodes *Fasciola gigantica* (Lutz and Siddiqi, 1967), *Philophthalmus megalurus,* and *Echinostoma revolutum* (Cain, 1969a), each of which possess two globin forms of hemoglobin with molecular weights ranging from 15 to 17,000 daltons. The two globins found in metacercaria tissues are distinct from the globins of *A. ornata* coelomic cell hemoglobins based on spectral differences and on elution differences using ion exchange media.

Tritiated glycine was found in gel filtration fractions that contain metacercaria hemoglobins. This evidence, along with ion exchange elution differences, spectral and functional differences, strongly suggests that at least the globin portion of the metacercaria hemoglobin is produced in the animal. Because glycine is also used in the synthesis of the protoporphyrin ring (Guyton, 1986), the appearance of label in metacercaria hemoglobin may also indicate the *de novo* synthesis of heme. Alternatively, heme groups could be obtained directly from ingested host hemoglobins, making synthesis unnecessary. Cain (1969b) showed that *Fasciolopsis buski* did not produce heme, but did synthesize the globin portion of its hemoglobin. This species also ingests its host's hemoglobin and, therefore, has an exogenous supply of heme. On the basis of the available evidence, the origin of heme in the metacercaria hemoglobin is unresolved.

The metacercariae also have a large molecular weight heme protein fraction. This fraction elutes as two distinct peaks upon ion exchange chromatography that correspond to two consistent hemoglobin peaks present in the whole metacercaria lysate. These two heme proteins demonstrate the presence of radiolabel and are chromatographically distinct from all hemoglobin fractions of *Amphitrite* HMW hemoglobin, indicating that they were synthesized in the larvae. *Ascaris* possesses two hemoglobin fractions, one low molecular weight form found in the tissues and a high molecular weight aggregate in the perienteric fluid (Okazaki *et al.*, 1965, 1967). However, there have been no reports of a high molecular weight hemoglobin in a trematode. We are uncertain at this time whether the heme proteins of the metacercaria high molecular weight fraction are functional hemoglobins. It may be that the two heme protein peaks seen in

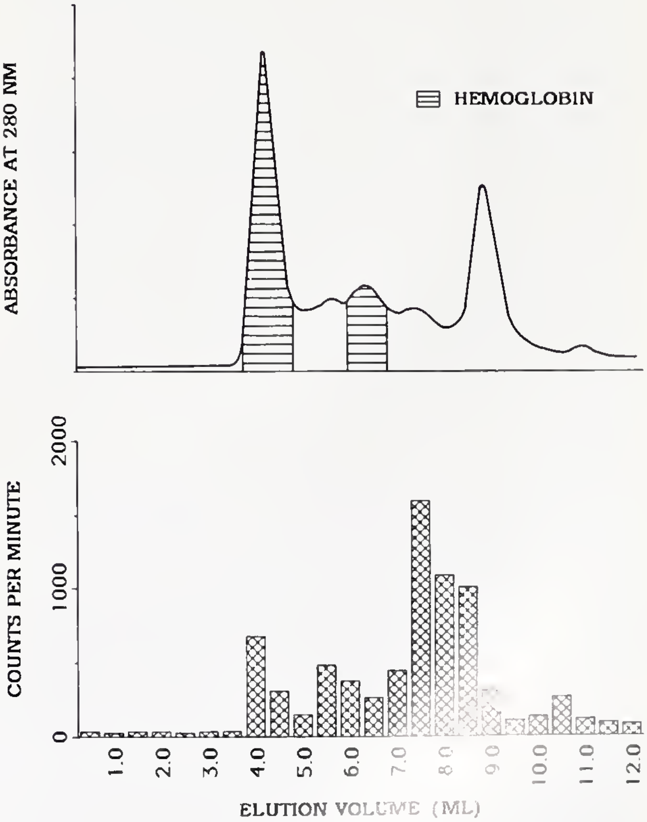

Figure 6. HPLC gel filtration elution profile for proteins from lysates of whole metacercaria incubated in H^3-glycine compared to a histogram of radiolabel (CPM) in sequential one-half ml samples of eluent.

the high molecular weight fraction are aggregations of altered forms of the two low molecular weight hemoglobins. Further research will be needed to determine the exact nature of this heme protein fraction from these animals.

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Induced Spawning of the Decapod Crustacean *Sicyonia ingentis*

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Abstract. The dark portion of the light/dark cycle initiated pre-spawning behavior in the penaeid shrimp, *Sicyonia ingentis.* Persistent swimming in the water column (pre-spawning behavior) correlated well with ovulation. Over 96% of the animals that exhibited active swimming behavior were ovulated as determined by the presence of green oviducts and/or their subsequent spawning. More than 87% of the ovulated animals responded to probing of the ovipores, located at the bases of the third pair of pereiopods, by immediately spawning. The ova from probe-induced spawns underwent a normal developmental sequence. A reliable and predictable technique for the assessment of ovulation and acquisition of viable ova from *S. ingentis* is described.

Introduction

Reproductively, decapods can be divided into those that brood their eggs (Pleocyemata) and those that are broadcast spawners (Dendrobranchiata). Present understanding of gamete interaction among the Dendrobranchiata is restricted to a few select species of penaeids (see Clark *et al.*, 1980, 1984). This is largely because researchers must rely on unpredictable natural spawns, a process that is a time consuming and uncertain operation.

Although information is scant, penaeid spawning appears to be correlated with the dark portion of the light/dark cycle. The majority of reports relating to the above are from studies of ovarian maturation and/or larval growth and development; they do little more than mention that gravid females spawned at some time during the night (Kelemec and Smith, 1980; Chamberlain and Lawrence, 1981). The most complete description of spawning concerns *Penaeus japonicus,* which spawns between the hours of 8:00 pm and 12:00 midnight (Hudinaga, 1942). *P. monodon* spawns between 8:00 pm and 6:00 am (Primavera, 1983; Motoh, 1981) and *P. trisulcatus* spawns between 8:30 pm and 10:30 pm (Heldt, 1938).

The penaeid *Sicyonia ingentis* can be inhibited from spawning if maintained under constant light (Griffin *et al.*, 1987); like other penaeids it spawns at night in a dark environment. Anderson *et al.* (1985) suggested that spawning in *S. ingentis* occurs after a period of swimming and that this prespawning behavior may be correlated with ovulation. These suppositions were based upon only a few observations. In the current report we document both the prespawning and spawning behavior of *S. ingentis* and describe a reliable and predictable technique for: (1) assessment of ovulation and, (2) immediate induction of spawning in ovulated animals.

Materials and Methods

Specimens of *Sicyonia ingentis* were collected off the southern California coast in otter trawls during the summer of 1987 and transported to the Bodega Marine Laboratory in chilled (8–10°C) seawater. Gravid females were isolated and placed in a 1000 gallon rectangular glass aquarium supplied with flow-through seawater at ambient temperature (10–14°C). Animals were fed *Artemia salina* and maintained under constant light.

To trigger prespawning behavior, the animals were placed in a dark environment (no lights at night; aquarium covered with black plastic sheet during either late morning or early afternoon). Animals in darkness were monitored with flashlights. To document prespawning and spawning behavior, some animals were allowed to

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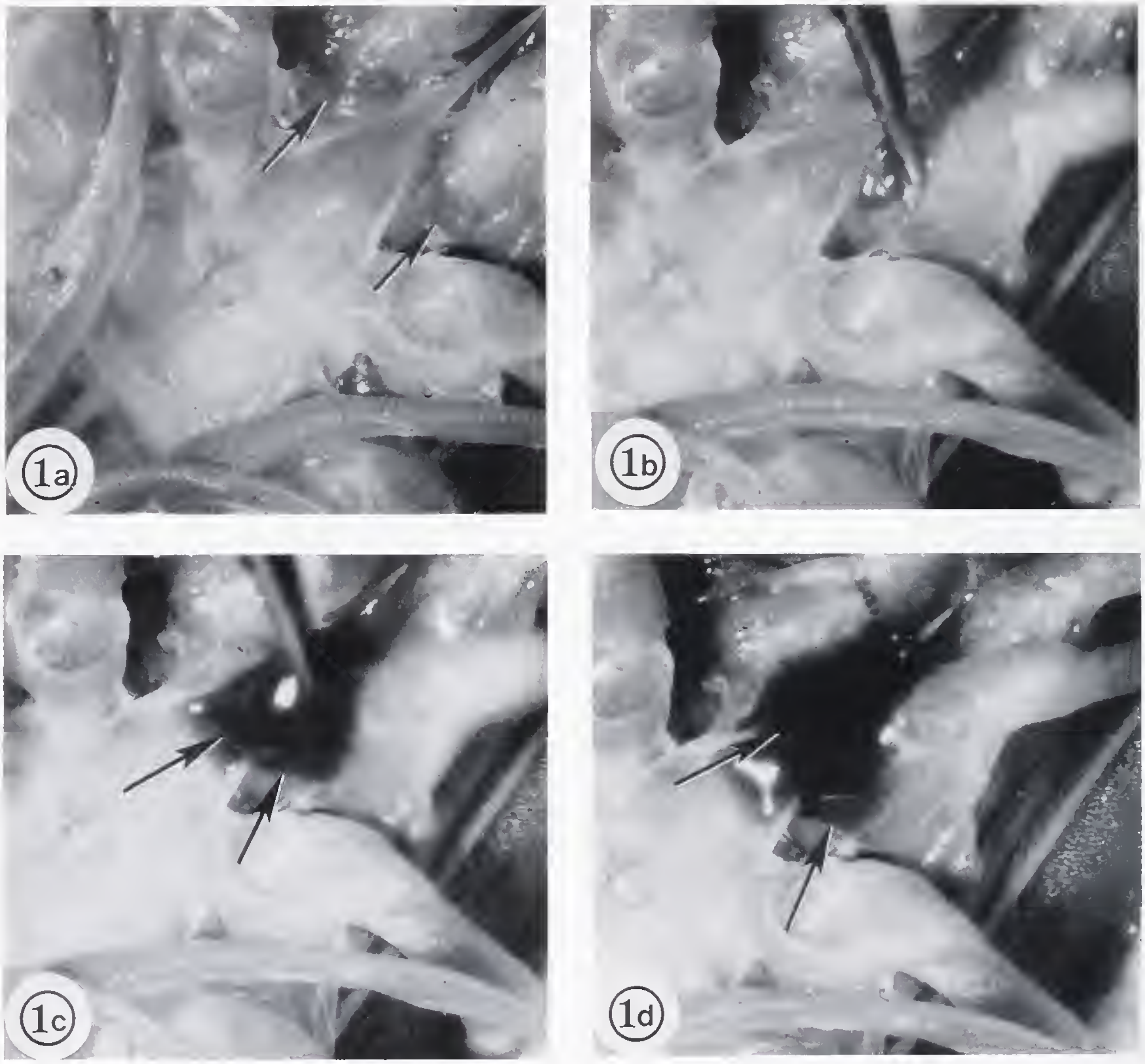

Figure 1. Ventral view of a female *Sicyonia ingentis.* 1a. Ovipores (arrows) located at the bases of the third pereiopods. 1b. The tip of the forceps are at the right ovipore. 1c. Forceps inserted into the right ovipore. The dark patch (arrows) is ova that have been released as a result of opening the ovipore. 1d. Ova have been released (arrows) from both ovipores as a result of opening the right ovipore. (5×)

complete spawning in the aquarium. Others were removed, the coloration of the oviducts was noted, and one ovipore was opened (probed) with forceps. If the probed animal released ova (spawning), she was placed on a 250 ml glass beaker of seawater and observed. We then answered the following questions: (1) did the female continue to spawn?; (2) were ova released from one or both ovipores?; (3) was spawning behavior normal compared with animals that had been allowed to spawn in the aquarium without manipulation?; and (4) were the ova from the probe-induced spawn fertilized? If the probed animal did not release ova, ovarian samples were excised and examined to determine if ovulation had occurred. As a result of ovulation (the loss of follicle cells from the oocytes) ova lay free in the ovary (Anderson *et al.*, 1984). Thus, ovulated and unovulated animals were easily distinguished when the ovary was dissected.

To determine if ova spawned from probed animals had been fertilized, ova were collected in 250 ml glass beakers of seawater and gently agitated for 30 min to keep the cells in suspension, incubated for 3 hours (at 20°C), fixed in seawater buffered 3% glutaraldehyde, and

Table I

Relationship between coloration of oviducts, ovulation, and induction of spawning

	Total number of animals	Green oviducts	Ovulated*	Probe spawns	Normal spawning behavior
Swimmers	65	63	60	57	53
Non-swimmers	28	4	3	2	0

* Ovulation was detected either by subsequent spawning (upon probing) or by dissection of the ovaries.

scored for percent normal cleavage. Fertilized ova undergo a normal (equal) cleavage whereas unfertilized ova exhibit an abnormal (unequal) pattern (Pillai and Clark, 1987). Control animals were allowed to initiate spawning naturally (without being probed) before they were placed on beakers filled with seawater. Percent cleavage in ova from these animals was compared with ova from probe induced spawns.

To determine if probing affected the time between first swimming and spawning, some females that initiated swimming behavior were placed individually in separate rectangular tanks containing flow-through seawater, kept in the dark as described above, and monitored for natural spawning. These animals were checked every 10 minutes for spent ovaries. The time of spawning, if it occurred after transfer to the tanks, was noted. The animals that had not spawned after fifty minutes were removed from the tanks and were probed to induce spawning as described above.

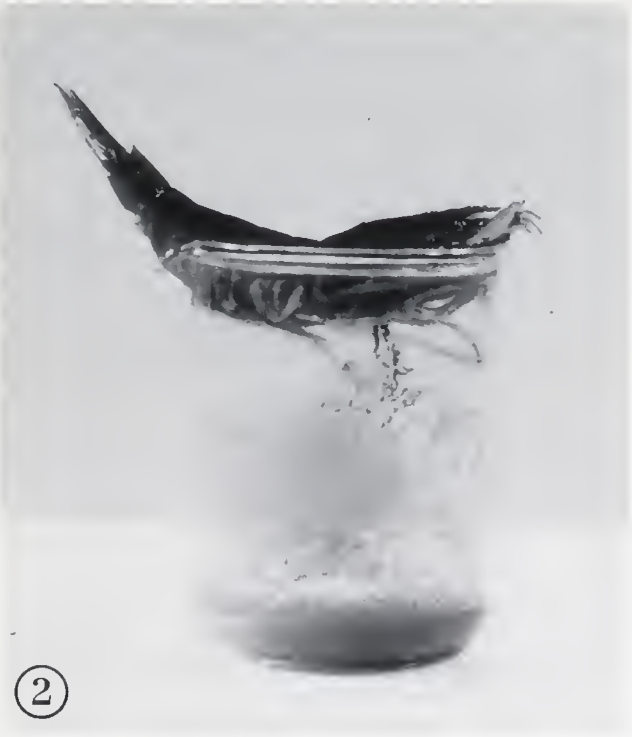

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Figure 2. The animal in Figure 1 after being probed at the right ovipore and placed over a 250 ml glass beaker containing seawater. Note the ova being spawned from both ovipores. (0.7×)

Results and Discussion

The manipulation of light cycles dramatically affected spawning in *Sicyonia ingentis*. Within 30–45 minutes after starting a dark cycle animals became active; they repeatedly rose into the water column and fell back to the bottom of the aquarium. Animals that maintained this active swimming behavior eventually remained at the surface and spawned after a variable amount of time. Such swimming activity at night has been described as a prespawning behavior in penaeids (Motoh, 1981; Anderson *et al.*, 1984). The present observations with *S. ingentis* indicate that a dark environment, regardless of the

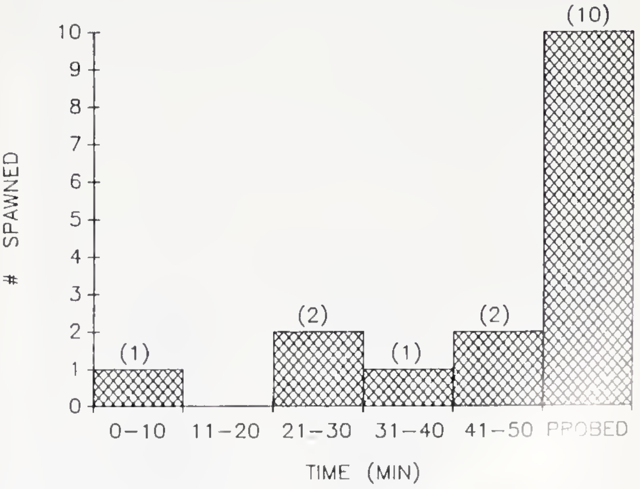
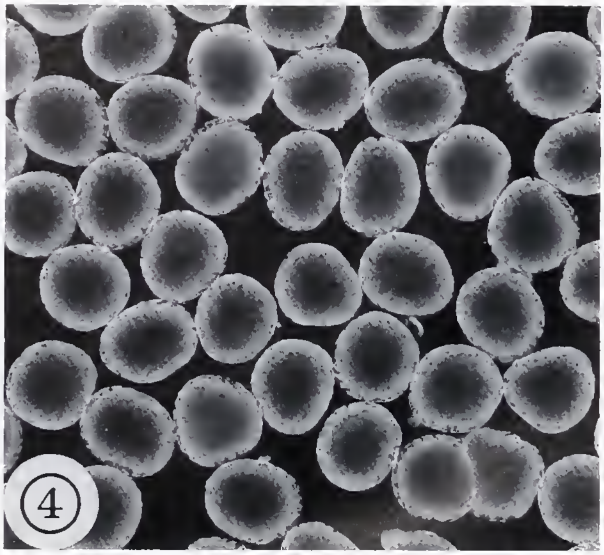


Figure 3. Graph illustrating that natural spawning does not immediately follow ovulation and that the time delay is variable. Animals that had ovulated (those exhibiting prespawning behavior) were placed in individual tanks and monitored at 10 min intervals to determine if spawning had occurred. Those that did not spawn within 50 min were probed. Numbers in parentheses represent the number of animals that spawned during each time period or after probing. Total n = 16.



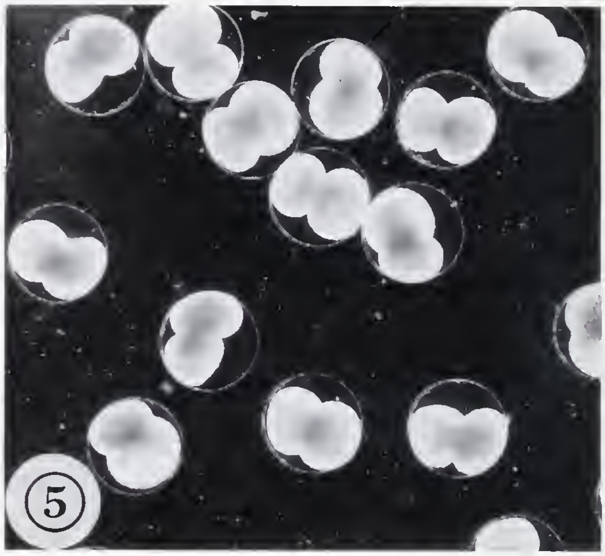


Figure 4. Dark field micrograph of ova from *Sicyonia ingentis* that was induced to spawn by probing at the ovipore. The ova were collected directly into fixative (seawater buffered 3% glutaraldehyde) at spawning. (50×)

Figure 5. Dark field micrograph showing two-cell embryos cultured from an induced spawn. Note the equal cleavage pattern. (50×).

time of the day, initiates prespawning swimming behavior and subsequent spawning.

Spawning behavior in *S. ingentis* is similar to that described for *Penaeus japonicus* (Hudinaga, 1942) and *Penaeus monodon* (Motoh, 1981). During spawning, the animal's pereiopods are held together and projected anteriorly while the pleopods are moved vigorously. Ova are released in a stream from the paired ovipores located at the bases of the third pair of pereiopods and mixed externally with sperm that are ejected from the thelycum (Clark *et al.*, 1984).

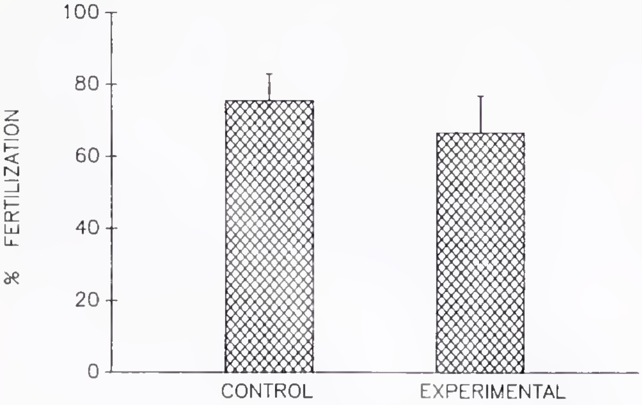


Figure 6. Graph illustrating the percent fertilization and normal development (up to second cleavage) of ova from natural spawns (control) and induced spawns (experimental). The two groups were not significantly different ($P > 0.1$, *t* test).

Ovulation, which precedes spawning in *S. ingentis* by an unknown period of time (Anderson *et al.*, 1984), correlates well with a change in oviduct color. The oviducts of ovulated females are green; the oviducts of unovulated animals are pale if not indistinguishable. Upon ovulation, free ova move down the oviducts producing a greenish appearance that is visible with the naked eye. Over 96% of the swimmers possessed green oviducts and more than 95% of these animals were ovulated as determined by subsequent spawning (upon probing) or dissection of the ovaries (Table I). Only 4 of 28 (14.3%) animals removed from the bottom of the aquarium (non-swimmers) possessed green oviducts. Two of these animals had completed ovulation and one had partially ovulated (Table I).

Animals that possess green oviducts and exhibit prespawning swimming behavior can be induced to spawn by probing an ovipore (Figs. 1a–d). Over 87% of such animals responded to probing of the ovipores by immediately spawning. More than 92% of this group of animals exhibited normal spawning behavior (Table I) as described above (see Fig. 2). Probing, however, had little effect on animals that did not exhibit pre-spawning behavior (non-swimmers). The only non-swimmers that responded to probing were the two animals that had completed ovulation; however, neither animal exhibited normal spawning behavior nor completed spawning (Table I). These data suggest that two criteria are necessary for successful probe spawns: ovulation and the initiation of prespawning behavior.

Probing one ovipore, regardless of the side of the animal, resulted in release of ova through both ovipores. Probing mechanically opened an ovipore. Although probing experiments demonstrated that spawning can be induced in ovulated swimmers on demand, they did not delineate whether probing decreases the time between ovulation and spawning. Of the 16 ovulated swimmers that were placed in individual tanks and monitored for natural spawning for a period of 50 min, only 6 (37.5%) spawned. The remainder of these animals immediately spawned upon probing (Fig. 3). These results demonstrate that once an animal has undergone ovulation and initiated prespawning behavior, it can be induced to spawn on demand even though the natural time to spawning may be variable.

Induced spawning does not significantly affect zygote quality. Both zygotes from induced and natural spawns were cultured through the first mitotic division (Figs. 4 and 5). Unfertilized ova would have exhibited abnormal (unequal) cleavage in contrast to the normal (equal) cleavage pattern in fertilized ova (Pillai and Clark, 1987). The percent fertilization in the induced spawns (65.5%) was not significantly different from that in natural spawns (75.5%) (Fig. 6). It would not have been unreasonable to expect lower fertilization rates in ova from induced spawns since females induced to spawn released ova prior to being transferred to beakers and initiating normal spawning behavior. Recent evidence indicates that such behavior is required for proper sperm-egg mixing (unpub. data).

This study demonstrates that ovulated animals can easily be selected and induced to spawn on demand under laboratory conditions. It is not known how the mechanical stimulus provided by probing the ovipores induces spawning. Stretch receptors may be involved, but to date this is only speculation. Further studies may explain this phenomenon in more detail. Finally, it is hoped that this technique will advance gamete research with decapod crustaceans.

Acknowledgments

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A Summary of the Symposium on "Gamete Dialogue in Fertilization: From Sea Urchin to Human"—in Memory of Alberto Monroy[1]

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Introduction

Alberto Monroy's scientific passion was to understand that initial interaction resulting in the union of sperm and egg and concomitant activation of development. He was an internationalist who involved scientists from around the world in intense conversation and experimentation around this phenomena. In recognition of his personal contributions to this understanding, and for his critical sparking of interest of so many other scientists in gamete interaction, more than 200 scientists gathered in Woods Hole for a two-day symposium to honor the memory of this remarkable person. The symposium was titled "Gamete Dialogue in Fertilization: From Sea Urchin to Human." Appropriately, the meeting sparked that open scientist-to-scientist dialogue that so marked Alberto Monroy's persona.

The title also reflected the two recurring themes of the program. The first was the reciprocal dialogue between sperm and egg and egg and sperm during the fertilization process and the second was the similarity of the fertilization process in sea urchins, tunicates, amphibians, and humans. To be sure, differences exist, but one came away with the generalization that there were common principles, even though the principals or agents involved in the fertilization process were variable.

Egg to Sperm Dialogue

A number of the talks considered the physiology of sperm. Many of these asked how small molecules released from eggs, egg jelly, cumulus, or *zona* regulate sperm behavior and the literal dance which ultimately results in sperm incorporation into the egg. Much of this initial work on fertilization began at the Marine Biological Laboratory in Woods Hole. Indeed, it was appropriate that these talks were presented in the Lillie Auditorium, named in honor of F. R. Lillie for his important contributions to fertilization physiology, especially as learned from studies on sea urchins.

David Garbers (Vanderbilt University) summarized his work and the work of scientists in Japan on the nature of the peptides (such as speract) released from sea urchin eggs that affect sperm physiology. Consequences of these peptides on sperm include dramatic changes in motility, respiration, ion fluxes, cyclic nucleotide metabolism, and behavior.

Garber's recent work has focused on isolating and characterizing the peptide receptor. Various peptide analogs can be synthesized and derivatives with substituted tyrosines remain active, allowing the tyrosine residues to be iodinated and cross-linked to the membrane receptor. These studies show that the receptor for speract in *Strongylocentrotus purpuratus* sperm is identical to guanylate cyclase. This enzyme is a major protein in the plasma membrane of sperm and its changes upon interacting with egg jelly have been elegantly described in work from both Vacquier's lab in La Jolla and Garber's in Nashville. An intriguing observation of Garber's recent work is the finding of considerable sequence homology of the speract receptor with atrial naturitic factor receptor (which similarly affects cyclic-GMP levels).

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[1] This symposium, held at the Marine Biological Laboratory, Woods Hole, Massachusetts on 24 and 25 August, 1987, marked the centennial of the founding of the Marine Biological Laboratory (1888–1988) and of the National Institutes of Health (1887–1987). It was sponsored by the Reproductive Sciences Branch of the National Institutes of Child Health and Human Development, the Rockefeller Foundation, and the Marine Biological Laboratory.

Correspondence to Dr. Epel.

Similar motility changes occur in sperm of other species as they approach the egg and in some cases these are also induced by peptides, as in horseshoe crabs and jellyfish. Mammals also use peptides, but occasionally use other small molecular weight compounds such as taurine and bicarbonate to regulate motility.

Don Wolf (Oregon Primate Center) summarized recent work on the hyperactivation of sperm in primates. This behavior, in which the sperm undergoes increased motility and rapid change of direction, was first observed in hamster sperm. Wolf described his work on this phenomena in primate sperm (both monkeys and humans) and his quantitation of hyperactivation by computer tracking programs. Recent research suggests a role of hyperactivation in fertility; for example, there is a correlation of the two in monkeys and humans.

An intriguing observation has arisen from observing hyperactivation as a function of time in capacitated sperm. Only a small percentage (3–20%) of these sperm exhibit hyperactivation at any time. Does this reflect the fact that only a small percentage of sperm are ever hyperactive or involved in fertilization? Or is there an inherent temporal variation in the sperm, with some sperm becoming hyperactive at different times? If the latter, could this reflect an adaptation inherent in the sperm population so that some sperm are always hyperactive, thus facilitating the fertilization process?

A second change that occurs in sperm, and which is also initiated by egg products, is the acrosome reaction. This can occur before sperm-egg binding or can be effected at the egg chorion/*zona* via some consequence of the sperm-egg binding. The limited knowledge available indicates considerable variation within the animal kingdom, even within the mammals, as to when this reaction occurs.

The two best understood systems, in terms of identification of the effector molecules, are gametes of starfish and mouse. Motonori Hoshi (Tokyo Institute of Technology) described his group's work on the nature of the substances that induce the acrosome reaction in the starfish. The major effector, a high molecular weight acrosome-reaction-inducing substance (ARIS) is a glycoprotein of greater than 10 million molecular weight. The active factor is most likely a carbohydrate moiety, since a pronase-digested fragment is active.

Other small molecular weight compounds are also necessary, and these co-factors are referred to as Co-ARIS. One of these is a sulfated glycosylated steroid or saponin. Chemical studies indicate that the sugar moiety is not important but the side change on the sterol is critical and sulfation is necessary. Recent studies have also identified a second co-factor, a peptide present in the egg jelly, apparently involved in initiating pH changes in the sperm which are a prerequisite for the acrosome reaction.

What is the situation in higher forms? In the mouse, a protein involved in sperm-binding to the *zona pellucida* also induces the acrosome reaction. Stanley Meizel (University of California, Davis) has examined the induction of the acrosome reaction in humans. He found that a factor extracted from human follicular fluid induces the acrosome reaction of human sperm. The factor is a 50,000 molecular weight glycoprotein and the active component is also a glycoside attached to the protein, as deduced from studies of various enzymes on the factor (for example, it is not sensitive to pronase and a small molecular weight active factor is released by an endoglycosidase).

Nicholas Cross (University of California, Davis) examined the interaction between sperm and *zona* in the human. Do sperm first bind to the *zona* and then undergo the acrosome reaction (as in the mouse), or can an acrosome reacted sperm bind to the *zona*? Cross's work shows that both alternatives can occur; sperm can bind to the *zona* and then undergo an acrosome reaction, but acrosome-reacted sperm can subsequently bind to the *zona*. This work is consistent with an *in vivo* role for Meizel's finding that a soluble factor from human follicular fluid induces the acrosome reaction. Cross and Meizel's work represent a technical *tour de force* because of the limited amount of material and reveal some differences between mice and man.

The third question addressed at the meeting concerned the nature of sperm-egg binding. A highlight was the report by Rosario DeSantis (Stazione Zoologica, Napoli) on sperm-egg binding in ascidians. Her paper summarized some of the major work that Alberto Monroy was engaged in during the latter part of his scientific career, and brought up the provocative observation of alternative mechanisms of sperm-egg binding among various organisms. De Santis described the research that led to the development of a simplified model for studying sperm-egg interaction, using vitelline coats isolated from glycerol-extracted eggs. Under these conditions, the vitelline coat retains species-specific and individual-specific sperm binding. The binding is prevented by high concentrations of L-fucose, suggesting that a fucose moiety is involved in sperm-egg binding. The dissolved vitelline coat does indeed have fucose-containing proteins. These proteins were isolated by chromatography on lectin columns; when added back to the sperm, the fucose-containing proteins prevented sperm-egg binding. The activity was retained in the carbohydrate fraction (*i.e.*, in pronase digests); this fraction prevented binding but did not induce the acrosome reaction. This suggests there may also be a component in the vitelline coat that induces the acrosome reaction.

De Santis and her colleagues recently isolated the complementary or vitelline-layer binding protein on the sperm, which is localized to the sperm tip and can be assayed by its binding to concanavalin A. This protein may have an alpha-fucosidase activity. It was suggested that the sperm-egg binding activity may occur through the tight dissociation constant of this enzyme. This novel mechanism has also been suggested for a galactosyltransferase activity apparently involved in sperm-egg binding in the mouse.

The temporal relationship between acrosome reaction and sperm-egg binding in the mouse is (1) binding and then (2) induction of the acrosome reaction. Bayard Storey (University of Pennsylvania) described current work from his group on the nature of the signal from the *zona* to the sperm that causes the acrosome reaction. Storey's group has made two interesting observations. The first answers the question of when—following sperm-egg binding—does the acrosome reaction occur? It was known, from looking at populations of bound sperm, that the acrosome reactions in the *population* of sperm slowly occurred over a period of several hours. They have now followed *individual* sperm bound to the *zona* and find that the acrosome reaction in the individual sperm occurs rapidly. It requires only two minutes. This suggests that there is considerable population asynchrony in the induction of the acrosome reaction, and a preliminary statistical analysis suggests that this is stochastic and follows a Poisson Distribution.

Storey's second observation concerns the signal that results in the induction of the acrosome reaction. They find that the initiation of the acrosome reaction is more rapid if phorbol esters are added. This suggests that protein kinase C may be involved in the signaling between the egg-*zona* and sperm in inducing the acrosome reaction.

A clue to the nature of the protein on the sperm which interacts with the *zona* and is coupled to inducing the acrosome reaction may come from the elegant experimental approach of Pat Saling (Duke University). She has prepared monoclonal antibodies against epidymal sperm and several of these stain the acrosomal crescent. One of these antibodies prevents fertilization. The antibody does not affect sperm-egg binding, but appears to affect the induction of the acrosome reaction as a consequence of the antibody binding. The antibody is not affecting the physiological response of the sperm, since one can still induce an acrosome reaction upon application of ionophore A23187 to antibody-treated sperm. Saling has recently looked at this antigen during the maturation process of the sperm and finds that the antigen is present fairly early in differentiation, but there are suggestions of a difference in molecular weight of the antigen during the later development of the sperm. Her work suggests an interesting processing of this surface antigen must occur during sperm maturation.

Following sperm-egg binding and induction of the acrosome reaction, the sperm somehow is able to penetrate the egg envelopes, abut against the plasma membrane, and effect fusion. The mechanism of this penetration was broached by studies of Robin Harrison (Agricultural Research Council, Cambridge) who presented his recent studies on hydrolytic enzymes in ram sperm. Much of the emphasis in the past has been on acrosin, but Harrison pointed out convincingly that there are clearly other proteases. Harrison described the properties of several peptidases which show varying degrees of extraction from the cell by salts. The role of these enzymes is intriguing and future work on them and possible changes in intracellular location will be awaited with great interest.

Is there a sperm receptor in the egg plasma membrane? Work on mammals suggests that there may not be species-specific receptors, as evidenced by the famous *zona*-free hamster experiment which showed that hamster eggs can be penetrated by human sperm. Edward Carroll (University of California, Riverside) described experiments suggesting that this may also be the case with sea urchins. He has developed a new method for denuding sea urchin eggs so that the fertilization membrane can be easily removed after fertilization. Such eggs can be refertilized and he observed that refertilization was even easier with interspecies crosses than by refertilization with sperm of the same species.

Sperm to Egg Dialogue

The second part of the symposium considered the other half of the gametic dialogue, the responses of egg to sperm. These include egg responses that modify sperm-egg binding and the responses of the egg resulting in the activation of the developmental program.

Two talks concerned the modification of the outer egg coats as a prelude to fertilization or as a consequence of the fertilization process. Jerry Hedrick (University of California, Davis) reviewed his group's work on the changes in the vitelline envelope which result in the acquisition of sperm-egg binding ability during the ovulation process and the subsequent loss of this sperm-egg binding ability as part of the fertilization process. As the sperm passes through the *pars recta* of the frog oviduct, a vitelline envelope protein is modified by a protease so that sperm can bind to the vitelline layer. Following fertilization, this same protein is again modified but this time there is a loss of ability of sperm to bind to the egg. This latter change results in part from the binding of a lectin which is released from the cortical granules.

A cortical granule protein might also be involved in the post-fertilization modification of the *zona* in the mouse egg. Richard Schulz (University of Pennsylvania) reviewed his recent work which provides important tools for understanding the cortical granule exocytosis and the modification of the *zona*. Work from Wasserman's laboratory had previously indicated that one of the major *zona* proteins (ZP3) has both sperm binding and acrosome-inducing activity. The protein loses both of these functions after fertilization. Schulz's analysis of the egg activation response suggests that there are different populations of cortical granules which respectively modify two of the three *zona* proteins. Also, the exocytosis of these different populations of granules may be mediated by separate intracellular signals.

Schulz finds that treatment of eggs with phorbol esters results in changes in the ZP3 protein so that the ability to bind sperm is retained, but its ability to induce the acrosome reaction is lost. Conversely, if eggs are injected with IP_3 (inositol trisphosphate), the protein that binds to acrosome-reacted sperm (ZP2) is altered, but there is no affect on ZP3 (which binds to acrosome-intact sperm and then induces their acrosome reaction). These results suggest that protein kinase C activity releases one type of cortical granule and that elevated calcium releases another granule. The data also suggest that there may be differences in mechanisms of egg activation between the mouse and sea urchin, centering on the nature of propagation of the cortical reaction (see below).

Three very interesting papers discussed the mechanism of sea urchin egg activation by sperm. Previous work from Jaffe's and Whitaker's laboratories (reviewed below) has provided strong evidence that polyphosphoinositide (PIP_2) hydrolysis is an initial concomitant of sperm-egg interaction and results in the post-fertilization release of calcium and other steps leading to egg activation. Laurinda Jaffe (University of Connecticut, Storrs) presented evidence indicating that this is via a GTP-binding protein, suggesting that phospholipase activation is tied to a G protein receptor complex. The evidence comes from examining the effects of injection of guanine triphosphate analogs; for example $GTP\gamma s$ results in parthenogenetic activation, whereas $GDP\gamma s$ prevents activation by the sperm. Cholera toxin and pertussis toxin effect activation and the eggs contain substrates which are ADP-ribosylated by these toxins. Cyclic nucleotides have no affect on egg activation, suggesting that the G protein effect is not via a nucleotide cyclase but most likely through phospholipase C.

This view is consistent with the idea that surface receptors are involved in egg activation and this concept is supported by experiments, on frog oocytes. In these oocytes, injection of IP_3 results in membrane potential changes which are mediated by an IP_3-induced release of calcium. Similar to the sea urchin case, injection of $GTP\gamma s$ also activates this current, indicating that G proteins are involved in the calcium release. Experiments suggesting that these G proteins can be coupled to plasma membrane receptors comes from recent studies by Jaffe in which mRNA from rat brain is injected into the *Xenopus* oocyte. This mRNA generates a serotonin receptor in the plasma membrane. If the oocytes are matured with progesterone and then serotonin is added to the oocytes, one gets the sequelae of fertilization identical to those induced with sperm. This elegant experiment suggests that a surface receptor can be coupled to G protein, resulting in egg activation.

Michael Whitaker (University College, London) presented evidence also consistent with the idea that PIP_2 hydrolysis is involved in egg activation. Work from his laboratory has shown a transient production of both IP_3 and diacylglycerol. The IP_3 induces calcium release, and the calcium used in egg activation apparently comes completely from endogenous stores; injection of IP_4 does not induce an influx of exogenous calcium needed for activation. Most interesting are experiments in which eggs are injected with high concentrations of neomycin to prevent PIP_2 hydrolysis. Under these conditions, sperm are not incorporated into the egg, consistent with the idea that either PIP_2 hydrolysis and/or calcium release is required for the incorporation. The idea that PIP_2 hydrolysis—as opposed to calcium release—is essential for sperm uptake comes from experiments in which eggs are injected with EGTA and then inseminated. Sperm-egg fusion occurs in these eggs. A calcium release does not occur but PIP_2 hydrolysis presumably has taken place, suggesting that this hydrolysis and its products are involved in the sperm-egg fusion.

Interestingly, Whitaker found that low amounts of cyclic GMP (cGMP) activates the egg. He suggests that perhaps this nucleotide is produced by the sperm (via the sperm guanyl cyclase?) and that introduction of cGMP is involved in the initial activation of the egg. One scenario is that cGMP activates a phospholipase (via a G-protein) with subsequent production of IP_3. There is then IP_3-induced calcium release and a subsequent Ca^{+2}-mediated PIP_2 hydrolysis which is responsible for propagation.

David McCulloh (University of Miami) used a novel electrophysiological approach to look at the question of when sperm-egg fusion occurs in relation to the electrical events of fertilization. His results suggest that sperm-egg fusion is coincident with the initial depolarization. Since calcium release does not occur until 10–15 seconds later, these results are consistent with the hypothesis that sperm-egg binding does not initiate receptor-mediated PIP_2 hydrolysis. PIP_2 hydrolysis might ensue from the injection of some activating "something" from the sperm or from "sperm" binding to a cytoplasmic receptor which then initiates PIP_2 hydrolysis

McCulloh and Edward Chambers, (University of Miami) used a loose patch clamp pipette technique to monitor sperm-egg fusion in *Lytechinus variegatus*. They introduce sperm through the patch pipette and monitor the capacitance change in the membrane under the pipette as an index of sperm-egg fusion. With another microelectrode, they monitor egg plasma membrane potential as an index of the electrical change accompanying sperm-egg interaction. Their results indicate that both events are coincident. Thus, sperm-egg fusion (at least as monitored by this technique) occurs within 1–2 seconds of sperm binding and hence is very early and significantly precedes the timing of the calcium rise. Using similar techniques but rapidly fixing eggs and then looking for electron microscope evidence of sperm-egg fusion, they (Frank Longo—University of Iowa—in collaboration with the Miami group) saw morphological evidence for fusion 5–8 seconds after the depolarization. Thus, several steps may be involved in the fusion process, an early one indicated by a capacitation change and a later one by morphological criteria. Irrespective, fusion measured by both of these methods significantly precedes the calcium release and suggests a sequence of events involved in the activation process.

Giovanni Giudice (University of Palermo, Italy) prefaced his scientific report with comments about the important contributions of Alberto Monroy to science in Italy and informed the audience that the Department of Experimental Embryology at the University of Palermo has been renamed the Alberto Monroy Laboratory. He then used the opportunity to describe the work in this Laboratory, primarily focusing on later development. One example is the intriguing research program on the control of mitochondrial DNA synthesis. Mitochondrial synthesis is active during oogenesis, is turned off by maturation, and does not begin again until quite late in development. Their work shows that the nucleus has an inhibitory affect upon mitochondrial DNA synthesis; enucleation, for example, results in an abrupt turn-on of DNA synthesis. Another area of research is on the mechanism of reaggregation of embryos and the types of molecules involved in the adhesion of the cells.

Much of the work of the Alberto Monroy Laboratory is concerned with macromolecular synthesis during development. Some research focuses on heat shock proteins and other research centers on the expression of a collagen gene which is similar to that seen in vertebrates and which begins to appear in the late blastula stage. Finally, a large research group is interested in histone synthesis and the nature of the molecules that control the formation of these important proteins.

Besides recounting the exciting advances in fertilization that have occurred over the last few years, the meeting was also marked by poignant tributes paid to Alberto Monroy. These included comments from some of his collaborators, such as the opening remarks by Charles Metz (University of Miami) and later remembrances by Leonard Nelson (University of Toledo). There were also greetings and comments on the importance of this work and the Symposium from Duane Alexander (Director of the National Institutes of Child Health and Human Development) and welcoming remarks and comments on Dr. Monroy and his relations with the Woods Hole community from the new Director of the Marine Biological Laboratory, Harlyn Halvorson. The meeting closed with a summation of the talks by David Epel (Hopkins Marine Station) and comments on clinical implications of this work from the major organizer of the meeting, Luigi Mastroianni Jr. (University of Pennsylvania).

Scientist–Scientist Dialogue

Looking broadly at these two days, two major new themes could be discerned. The first centered on the question of whether fertilization is a "once in a lifetime" phenomena or involves biological principles that are repeated over and over again during the life history of the organism. In the past, many workers have emphasized fertilization as an example of general cell biological phenomena. This is indeed the case for some events, such as cortical granule exocytosis, which clearly involves calcium as in other systems and the mechanisms of signal transduction, which involve G proteins and PIP_2 hydrolysis as also seen in many somatic cells. However, it is also becoming evident that gametes are specialized cells that may have features unique to their function. An example is the question of whether there are plasma membrane receptors involved in signal transduction similar to growth factor receptors in other cells or whether there are unique aspects involving sperm-egg fusion.

A second and related theme, which also surfaced at this meeting, is the question of phylogenetic/evolutionary similarities of the fertilization process. Superficially, the sperm-egg dialogue is the same; the sperm is always small, the egg is always large; the egg releases substances that activate sperm; and the sperm-egg contact ultimately activates the egg. However, a review of the limited comparative knowledge we have, such as at this conference, shows some differences among the organisms. A theme that emerged, however, was that in spite of this diversity, the principles governing the phenomena are the same although the principals or actors in this dialogue vary. One example would be in the acrosome reaction. Clearly the nature of the inducers that are released by the egg, and which cause the acrosome reaction differ among organisms. They differ chemically, and they differ in origin—*e.g.*, the jelly layer in invertebrates, the cumulus or follicle cells in humans, and perhaps the *zona* in the mouse. Yet, the underlying response, mechanisms, and general principals are the same; only the characters differ.

The conference was a provocative exposition of the theme of gamete dialogue and it also resulted in important dialogue but between the participants. It was a conversation that Alberto Monroy would have relished.

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Reference: *Biol. Bull.* **174:** 191. (June, 1988)

"To See What Everyone Has Seen, To Think What No One Has Thought"

—ALBERT SZENT-GYÖRGYI

A symposium in his memory and honor

Lillie Auditorium
Marine Biological Laboratory
Woods Hole, Massachusetts

1 and 2 July 1988

Friday, 1 July

8:30–9:00	Remarks*: Denis Robinson **Benjamin Kaminer** (Boston University School of Medicine) "On Szent-Györgyi"
9:10–9:50	Remarks: Laszlo Lorand **Michael S. Brown** (University of Texas) "Toward a Molecular Understanding of Cholesterol and Atherosclerosis"
10:10–10:30	Break—Refreshments
10:30–11:10	Remarks: Annemarie Weber **Joseph Goldstein** (University of Texas) "Toward a Molecular Understanding of Cholesterol and Atherosclerosis" (continued)
11:25–12:05	Remarks: George Wald **Michael Berridge** (AFRC, Cambridge) "Inositol Lipids and Cell Signalling"
12:20–1:25	Lunch
1:30–2:10	Remarks: Ferenc Guba **Gottfried Schatz** (University of Basel) "How Proteins Move From the Cytoplasm Into Mitochondria"
2:25–3:05	Remarks: Severo Ochoa **Aaron Klug** (MRC Laboratory of Molecular Biology, Cambridge) " 'Zinc-Fingers'—A Novel and Ubiquitous Protein Motif for Nucleic Acid Recognition"
3:25–3:50	Break—Refreshments
3:50–4:30	Remarks: Andrew Szent-Györgyi **Hugh Huxley** (Brandeis University) "Finding Out How Actin and Myosin Produce Movement" Remarks: F. Bruno Straub Setsuro Ebashi
5:00–6:00	Refreshments
8:00–9:00	Remarks: Benjamin Kaminer **Daniel E. Koshland Jr.** (University of California, Berkeley) "The Future in Biological Research: What is Possible and What is Ethical"
9:15	Wine and Cheese

Saturday, 2 July

9:00–9:40	Remarks: Salvador E. Luria **David Baltimore** (Whitehead Institute, MIT) "The Molecular Basis of Differential Gene Transcription"
9:55–10:35	Remarks: John Gergely **Philip Leder** (Harvard Medical School) "The Cancer Problem: An *In Vivo* Approach" Remarks: Teru Hayashi
11:00–11:20	Break—Refreshments
11:20–12:30	Remarks: Ronald Pethig **Carlton Gajdusek** (NINCDS/NIH) "Transmissible and Non-Transmissible Dementias as Brain Amyloidoses" Remarks: Zolton Bay Tamas Erdos Closing Remarks: Prosser Gifford

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* Remarks will be on Albert Szent-Györgyi.

Symposium Committee: Benjamin Kaminer and Denis Robinson, co-chairmen; Peter Gascoyne; Laszlo Lorand; Jane McLaughlin, Andrew Szent-Györgyi; and Richard Whittaker (*ex-officio*).

Support from Hoffman-LaRoche, Inc. and the Alix Robinson Memorial Fund is greatfully acknowledged.

Reference: *Biol. Bull.* **174:** 192–213. (June, 1988)

Abstracts of Symposium Lectures

On Albert Szent-Györgyi

Benjamin Kaminer

Boston University School of Medicine

Albert Szent-Györgyi, born in Budapest on September 16, 1893, died at the age of 93 at his home in Woods Hole on October 22, 1986. The story of the major portion of his life is best portrayed by Szent-Györgyi himself in his inimitable and delightful style in his autobiographical sketch "Lost in the Twentieth Century" (*Annual Reviews of Biochemistry,* 1963). The article is reprinted in this publication as is his bibliography. On presenting the Nobel Prize in Physiology and Medicine to Szent-Györgyi in 1937, Professor E. Hammerstein referred to him as the "new conquistador from Szeged"—who "conquered new ground by intuitive daring and skill"—with a "clear vision for essentials." Many aspects of his approach to science and his humanism which had a profound impact are recalled in this symposium.

"Prof" (as Szent-Györgyi is affectionately known to many) is remembered particularly for three major scientific contributions: on the catalytic role of C4-dicarboxylic acids in the oxidation process of pigeon breast muscle (a material with a high oxygen consumption that he first introduced for metabolic studies); on the isolation and identification of Vitamin C; and on the foundations he laid for the molecular basis of muscle contraction.

A few less-known early publications are worth recalling because of their predictive significance and current developments. In a paper with Brinkman (1923), an insightful conclusion, based on simple observations, was reached on hemoglobin being "attached to the surface of the stromata" of red blood cells a consideration currently being pursued particularly with reference to sickle cell anemia. Following studies on crude extracts of heart muscle effecting its activity, Drury and Szent-Györgyi (1929) identified and crystallized adenylic acid as the active principle. They showed that it and adenosine influenced the conduction of heart muscle and decreased the blood pressure of animals by dilating vascular smooth muscle. Adenine compounds are now being recognized as putative neurotransmitters. Based on his conviction that basic processes of life will be the same in all cells and tissues, Szent-Györgyi, in an article titled "On protoplasmic structure and function" (1940) wrote "a protein fraction analagous to myosin [*i.e.,* myosin B, prior to the discovery of actin] should be found in any cell." He then described the extraction of a protein from kidney, liver, brain, nerves, mammary gland, parotid, lymph gland, whole embryos, Rous sarcoma, and Ehrlich carcinoma. The protein from these sources behaved like myosin and had a rod-like shape. Szent-Györgyi, ahead of his time, thus anticipated the discoveries of myosin and actin in non-muscle cells.

Looking further ahead, at a time of great productivity on muscle proteins in his laboratory in Hungary, Prof with his Dyonisian, intuitive approach speculated on energy levels in proteins and on "a new period in biochemistry, taking this science into the realm of quantum mechanics" (*Nature,* 1941). He organized joint seminars with theoretical physicists, stimulated the interests of his associates in this direction, and paved the way in advance for his eventual digression from muscle research in the mid-fifties at his Institute for Muscle Research at the Marine Biological Laboratory in Woods Hole. He vigorously pursued studies on fluorescence, phosphorescence, and charge-transfer reactions. In 1959, when I joined his Institute, he purchased an electron spin resonance machine that Irvin Isenberg, a fine biophysicist, used to measure free radical formation in various reactants. Hence having challenged himself in the 1940's, he chose this general direction of submolecular biology in his quest to understand the normal "living state" and cancer until the end of his days; several months before his death, while ailing, he continued his research.

Prof found a haven at the MBL in 1947 and could not have functioned as he did in any other institution. And the MBL would not have been what it is today if not for Prof. He stimulated generations of summer students and investigators and had a profound impact on the Physiology course. His presence at the MBL attracted prominent scientists and his private summer conferences at his home influenced a number of physical chemists and physicists.

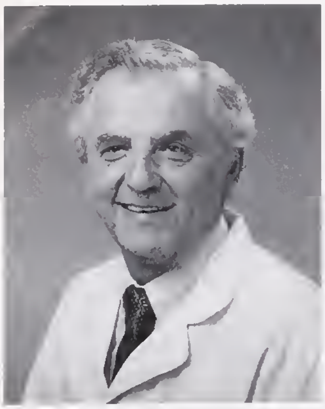
Intensely concerned with the serious problems of mankind, Prof stepped outside the confines of his laboratory, fearlessly expressing his philosophical and political viewpoints. In his little book *The Crazy Ape,* Prof sees hope in the youth of the world and calls upon them to organize and exercise their democratic power to create a new and better world.

Others in this symposium portray Prof's charm, vitality, and approach to science. I wish to emphasize that boyish, imaginative, and adventurous spirit which remained with him throughout his life and the exceptionally high personal moral and ethical standards that he set for himself.

In 1960 during the late summer, a tremendous storm developed with hurricane warnings. Prof's raft (anchored in the sea in front of his house) to which he swam every morning in the summer, was being swept away towards the rocks. He saw this while returning home for lunch and quickly developed a plan to save the raft. Naturally, Marta ("Profne" as Prof's wife was called) forbade him to even contemplate the idea of saving the raft and risking his life. While she was busy upstairs he quietly went to the phone, called me in the lab, and asked if I would come to his house to help him save his raft. I hopped onto my bicycle and before I reached his house ("the Seven Winds") Prof had already sneaked out of the house like a naughty, defiant school boy determined to fulfill his objective. Walking with lines and anchors around his shoulders towards the boat house, he outlined his strategy: he would drive his 15-ft outboard motor boat towards the raft (which was shaped like a square doughnut with a big hole in the middle). I was to cast a big anchor into the middle of the raft, hook it underneath, and then we would drag the raft away from the rocks towards its original position.

The 45-horsepower motor roared as we entered at full throttle into "The Hole." We hardly moved. But Prof managed to navigate though the tremendously powerful cross currents and high waves that were tossing and turning and filling the small boat with water. The progress was slow, and I thought surely he would soon turn back. But no; eventually we came close to the raft. The motor was put in neutral, and the boat was bobbing and tossing like a cork. We were dangerously close to the raft and the rocks. I tied the line to the stern cleat and eventually cast the anchor and successfully hooked the raft. Prof went full throttle ahead—but we hardly moved. After gaining some distance the anchor loosened from the raft. So we tried again, successfully casting and sending the boat full throttle ahead. We had moved some distance from the rocks when the raft's anchor apparently stuck; even with full throttle the boat didn't move and all of a sudden the boat swung around so that the line caught my right thigh against the boat. However hard I tried to hold onto the line I couldn't prevent it from crushing my thigh. That unforgettable feeling of rope "cutting" into my flesh led me to shriek and to instinctively topple overboard into the stormy sea thereby freeing my leg. My shriek must have reached his ears and he turned around and saw me in the sea clinging to the boat, lifting myself and tumbling into the boat. He cut down the throttle, thus reducing the tension on the line. The anchor loosened and I pulled it in; Prof dared not leave the controls as we were continuing to be swept and turned by the stormy sea. At this point I was sitting in a large pool of water (both gas tanks were floating!) and blood was oozing from my six-inch rope wound. Only then did Prof decide to give up, steering the boat back through the stormy Hole to the boat house; he had to sneak back into the house. The naughty boy did in fact save the raft and I still have a mark on my thigh. Prof's boyish, imaginative, and adventurous spirit also left its mark on the scientific world.

And finally, some words about his high standards of personal morality. When Prof isolated vitamin C from paprika he did not patent it. He sent the methods of purification and samples of the vitamin to scientists all over the world for further study. He also sent a large amount to the League of Nations for distribution to countries with a prevalence of scurvy. His efforts to raise money were always for the purpose of continuing the scientific efforts of his laboratory and his personal salary was always a modest one. This is epitomized particularly over the last eight years of his life when a foundation that had been formed to specifically support Szent-Györgyi's research was prospering and also supporting other scientists all over the world. Even as Scientific Director of the foundation, Szent-Györgyi again lived very modestly. He could not afford to heat his entire house in the winter, closing off sections of the house and he wore an insulated cold-room coat because he had turned down the thermostat to a low temperature (at his age!). Prof did not accumulate any savings and interestingly, never made arrangements for a retirement fund. I never discussed the latter with him, but I guess he deliberately or (subconsciously) did this as a safety device so as to never retire. Indeed he "died at the bench," a man devoted to increasing the wealth of knowledge while denying himself any personal wealth.



Benjamin Kaminer

Benjamin Kaminer, born in Slonim, Poland on May 1, 1924, immigrated at age 6 with his parents to Johannesburg, South Africa. He is currently Professor and Chairman of Physiology at Boston University School of Medicine, a position he has held since 1970. He received his medical degree in 1946 from the University of Witwatersrand in Johannesburg and after clinical internships went to London in 1949 for further training and research experience. On returning to South Africa temporarily, he joined the Physiology Department at Witwatersrand. Fascinated by Szent-Györgyi's questions and speculations on the possible role of water, the "*mater* of life" in muscle contraction, he embarked on a series of studies on muscle using heavy water, assuming that its properties different from normal water would help dissect out some mechanisms of muscle contraction. A Rockefeller Fellowship enabled him to join Szent-Györgyi's Institute in 1959. On arrival, having delayed publication of his results, he was hoping for help with interpretation from the master of muscle. After listening attentively to the findings, Szent-Györgyi simply pronounced "The more I know about muscle the less I understand it" (that naturally put Kaminer at ease) and he suggested that the interesting results be written up. Szent-Györgyi proceeded excitedly, his blue eyes sparkling, to explain simply his current ideas on charge transfer reactions. "A new dimension must be explored to understand the meaning of life" he exclaimed, and he suggested that some of his ideas had been sufficiently tested to try out on muscle. Kaminer worked year-round in the Institute for Muscle Research at the MBL until 1969 and has returned every summer since. He was Chairman of the MBL Instruction Committee, a Trustee, and a member of the MBL's Executive Committee. His current research is on calcium binding regulatory and storage proteins in sea urchin eggs and the squid nervous system.

Lost in the Twentieth Century[1]

Albert Szent-Györgyi

Laboratory of the Institute for Muscle Research
at the Marine Biological Laboratory,
Woods Hole, Massachusetts

It used to be said in my home town, that the cheapest funeral you could get consisted of taking a candle in your hand and going out, yourself, to the churchyard. As judged by the previous introductory articles, this one should be something like it: an obituary written by the fellow himself. Let it be that. I have no quarrel with the idea of completing my cycle of life. What goes against the grain is writing about myself, since I am averse to all forms of exhibitionism. Moreover, I like to look forward, not back.

Overlooking my case history, I find a complete dichotomy. On the one hand, my inner story is exceedingly simple, if not indeed dull: my life has been devoted to science and my only real ambition has been to contribute to it and live up to its standards. In complete contradiction to this, the external course has been rather bumpy. I finished school in feudal Hungary as the son of a wealthy landowner and I had no worries about my future. A few years later I find myself working in Hamburg, Germany, with a slight hunger edema. In 1942 I find myself in Istanbul, involved in secret diplomatic activity with a setting fit for a cheap and exciting spy story. Shortly after, I get a warning that Hitler had ordered the Governor of Hungary to appear before him, screaming my name at the top of his voice and demanding my delivery. Arrest warrants were passed out even against members of my family. In my pocket I find a Swedish passport, having been made a full Swedish citizen on the order of the King of Sweden—I am "Mr. Swenson," my wife, "Mrs. Swenson." Sometime later I find myself in Moscow, treated in the most royal fashion by the Government (with caviar three times a day), but it does not take long before I am declared "a traitor of the people" and I play the role of the villain on the stages of Budapest. At the same time, I am refused entrance to the USA for my Soviet sympathies. Eventually, I find peace at Woods Hole, Massachusetts, working in a solitary corner of the Marine Biological Laboratory. After some nerve-racking complications, due to McCarthy, things straightened out, but the internal struggle is not completely over. I am troubled by grave doubts about the usefulness of scientific endeavor and have a whole drawer filled with treatises on politics and their relation to science, written for myself with the sole purpose of clarifying my mind, and finding an answer to the question: will science lead to the elevation or destruction of man, and has my scientific endeavor any sense?

All this, in itself, would have no interest. There are many who did more for science, were braver, suffered more agony and even paid the penalty of death. What may lend interest to my story is that it reflects the turbulence of our days. So to give sense to my story I will have to start by asking: why all this trouble and what is its relation to science?

* * *

Erasmus of Rotterdam, that sage of the Early Renaissance, distinguished between calm and turbulent periods of human history which shows throughout the same repetitive story. Man develops a certain philosophy and creates the corresponding institutions and there is peace. Then his thoughts change and the new outlook has to fight the outgrown structure and thinking, with all its prejudice and vested interest; there is trouble till the new views predominate and the corresponding new order is established.

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The world has never known a more tumultuous period than ours and so, if Erasmus is correct, there has to be a correspondingly deep change in our ideas, a change more profound than any earlier one. It is clear to me what this change is: a transition from the prescientific to the scientific. Not only is this change profound, but it has come upon us too suddenly, leaving no time to adjust.

The difference between the two worlds is best illustrated by the story of the two stones, and of Aristotle, one of the greatest prescientific thinkers, and Galileo, one of the first modern scientists. Aristotle said that a big stone falls faster than a small one. The interesting point about this statement is not that it was wrong, but that it never occurred to Aristotle that he could try an experiment, to test his ideas. He would have considered such a proposal an insult. Man had only to think to find the truth, his mind being superior to crude experience. The mind reigned supreme. There was no reason to doubt, either, that what the senses conveyed was the last reality. If we touch things, they feel hard or soft, wet or dry; so, according to Aristotle, these had to be the ultimate elements out of which the world was built. There could not be the least doubt that it was the Universe which rotated around us. It was only a small additional step to suppose that even our feelings were trustworthy guides and that our everyday experience could be extended to problems beyond our reach. So if man resented death there could be no death, only Hades, Hell, or Heaven. If man wants a house, he has to build it, so if there is a Universe, somebody must have built it and be running it, somebody more powerful than ourselves. So man populated his world with gods, one or many, shaped in his own image. But even if there were beings more powerful than ourselves, we had to be their main concern, and remained the center.

So gradually, man built himself an imaginary world based on "faith," that is, accepting things without evidence. This faith was codified at different ages as religious in the name of which men tortured, subjugated, and killed one another. What underlay this prescientific thinking was man's trend for autistic thinking and his boundless self-confidence. While thinking himself the center, supreme master and judge, man had to remain the toy of Nature.

Two thousand years after Aristotle, something must have happened to man's mind for here and there people appeared, like Copernicus or Kepler, who modestly tried to put two and two together, while a boisterous young man went up a leaning tower to drop two stones, a big one and a small one, bidding his companions to observe which one hit the pavement first. What is essential about this simple act was a humble attitude: if we want to find out something about the world around us we have to ask questions modestly, that is, do experiments.

The same young man did not trust the perfection of his senses either, and later built the telescope to improve his sight. With his improved sight he could see the satellites of Jupiter and the rings of Saturn, never seen by man before, clearly indicating that the Universe could not have been created solely for man's pleasure or temptation. Today, three and a half centuries later, we see experimental science in rapid expansion, changing all parameters of human existence, creating an entirely new world, in which man has become the short-lived inhabitant of one of the small satellites of one of the millions of stars of one of the millions of galaxies, in a Universe expanding at increasing speed, dominated by quantum laws. What underlies this change is a new scientific thinking. The essential feature of this thinking is humility, the realization of our imperfections. The first command of this thinking is to accept nothing without evidence, face problems as such, with a cool head, without fear or prejudice, with uncompromising honesty of thought, unbiased by fear, hopes, or interest.

We are living in the middle of the transition from the prescientific to the scientific thinking, hence the "tumult." We still have God on our lips and our coins, but no more in our hearts. If we are taken ill we may still pray, but we take penicillin alongside. We pray for peace but heap up H-bombs for safety. We preach Christ and talk "overkill." This world is symbolized for me by the colossal statue of Christ, standing on a hill in Spain, stretching out His Arms to mankind, and wearing on His Head an enormous lightning conductor to protect Him, should the Almighty Father try to smite Him by lightning. We find the new expanding Universe a rather cold place and do not dare to abandon the old one. The trouble is that the two worlds cannot be mixed and the father inquisitor was right when he said to Galileo that "your teaching and the teaching of the church cannot exist side by side." We cannot build, unpunished, H-bombs by science either, and then run them with the XVIII Century egotistic, narrow, sentimental, and deceitful political thinking. It makes no sense to shoot astronauts out into space to reach other stars and erect ten-foot concrete walls to separate man from man. In its own time prescientific thinking did build a stable world, but science has irretrievably undermined the acquiescence in misery as the attribute of human existence, and has undermined the old hierarchies of gods, princes, barons, haves and have-nots, well-fed and hungry, developed and underdeveloped.

There is no way back, and we have to face squarely, the free choice between undreamed of wealth and dignity, and self-destruction which science has offered. My problem is: to what is science leading, and whether science can build a world in which man can feel, once more, at home? I will attempt to answer these questions at the end, after having given my case history.

* * *

On my Mother's side, I am the fourth generation of scientists. My Father was interested only in farming and so my Mother's influence prevailed. Music filled the house and the conversation at the table roamed about the intellectual achievements of the entire world. Politics and finance had no place in our thoughts. I am a scientist, myself, because at an early age I learned that only intellectual values were worth striving for, artistic or scientific creation being the highest aim. I strongly believe that we establish the coordinates of our evaluation at a very early age. What we do later depends on this scale of values which mostly cannot be changed later. We are somewhat like Dr. Lorenz' goose which has hatched at the foot of a chair and recognized the chair as its mother all its later life. This is important for education, in case we are not intending to produce only "corporation men" with their intellectual crew cuts.

I must have been a very dull child. Nothing happened to me. I read no books and needed private tutoring to pass my exams. Around puberty, something changed and I became a voracious reader and decided to become a scientist. My uncle, a noted histologist (M. Lenhossek), who dominated our family and was a precocious child himself, violently protested, seeing no future for such a dull youngster in science. When his opinion gradually improved, he consented to my going into cosmetics. Later, he even considered my becoming a dentist. When I finished high school with top marks, he admitted the possibility of my becoming a proctologist (specialist of anus and rectum; he had haemorrhoids). So my first scientific paper, written in the first year of my medical studies, dealt with the epithelium of the anus. I started science on the wrong end, but soon I shifted to the vitreous body, the fibrillar fine structure I explored with new methods.

I have mentioned this early history of mine because it suggests that no final judgment should be made of children at too early an age.

I must have achieved some reputation as a histologist when, as a third-year medical student, I became increasingly discontent with morphology which told me little about life. So, I shifted to physiology but had to break my studies for compulsory military service. World War I found me in uniform.

Centuries-old tradition told us Hungarians to ask no question when we were called upon to fight. I did accordingly, but during the first three years of the war I was gradually overcome by a burning desire to return to science. At the same time I became increasingly disgusted with the moral turpitude of military service. I could see clearly that we had lost the war and that we were being sacrificed senselessly by a ruling clique; the best service I could do for my country was to stay alive. So, one day, when in the field, I took my gun and shot myself through the bone of my arm. With all the deeply ingrained tradition this was quite difficult to do and it was also the more dangerous road. Anyway, it took me back to the capital where I got my M.D., after which I continued my service in a bacteriological laboratory of the army. Here, I got into trouble but once, when I objected to experiments, dangerous to life, done on Italian prisoners of war. Since the man responsible for these experiments had two stars more than I had, I was punished, and sent to the North Italian swamps where tropical malaria made life expectancy very short. A few weeks later the war collapsed and so I pulled out alive and returned to the laboratory.

I wanted to understand life but found the complexity of physiology overwhelming. So I shifted to pharmacology where, at least, one of the partners, the drug, was simple. This, I found, did not relieve the difficulty. So, I went into bacteriology, but found bacteria too complex, too. I shifted on, to physicochemistry and then to chemistry, that is, to molecules, the smallest units in those days. Ten years ago I found molecules too complex and shifted to electrons, hoping to have reached bottom. But Nature has no bottom: its most basic principle is "organization." If Nature puts two things together she produces something new with new qualities, which cannot be expressed in terms of qualities of the components. When going from electrons and protons to atoms, from here to molecules, molecular aggregates, etc., up to the cell or the whole animal, at every level we find something new, a new breathtaking vista. Whenever we separate two things, we lose something, something which may have been the most essential feature. So now, at 68, I am to work my way up again following electrons in their motion through more extensive systems, hoping to arrive, someday, at an understanding of the cellular level of organization. So the internal course of my life made a smooth sinusoid curve; not so the external course.

After the War, I became assistant at the pharmacological laboratory of the newly founded University in Pozsony, an old Hungarian town. A few months later Pozsony was given, by the Versailles Treaty, to Czechoslovakia (it is now called Bratislava) and we had to clear out. We saved our scientific equipment not without danger, getting it one night, dressed as workmen, through the closely guarded gates of the campus. Meanwhile, in Hungary, the communists took over, which meant a complete loss of all my belongings. At the very last moment, I rescued one thousand English pounds. These I shared with my Mother, whom I visited at Budapest. For such a visit the wintry Danube had to be crossed in a small overcrowded boat at night, at a point where there were no Czech patrols, who shot at sight. In my company was a nun, Sister Angelica, who was deadly frightened and clung to me desperately. On my return I had to spend a night in the snow and arrived in Pozsony with a grave pneumonia. I probably owe my life to the devoted nursing of Sister Angelica. After this, I took my wife and child and steered west. The English pounds allowed me to live, very modestly, for a little while, during which time I wanted to gratify my desire to do research. First, I went to Prague to learn some electrophysiology from Armin von Tschermak, from there to Berlin to learn about pH from Michaelis, (who later spent his last summer in my guest house at Woods Hole). From Berlin I went to Hamburg to the Institute for Tropical Hygiene. My calculation was that while I did research on physicochemical lines, I would learn enough about tropical medicine to be hired by some colonial government, once my money gave out. This time having arrived, I bought my tropical equipment, ready to go, but fate would have it that the Dutch Physiological Society held its meeting in Hamburg, and one of the participants was W. Storm van Leeuwen, professor of pharmacology in Leiden. He had with him Professor Fritz Verzar, serving as his associate professor. Verzar was about to return to Hungary so he introduced me to Storm van Leeuwen who invited me to take Verzar's post, which I took. After two years at Leiden, where I devoted my free time to learning chemistry, I joined Hamburger's Laboratory at Groningen where I worked for another four years. Salaries were very low but allowed for a very modest life, which was happy and quiet.

Now, I thought myself capable of tackling a biochemical problem. I embarked on biological oxidations. At that time a violent controversy raged between O. Warburg and H. Wieland and their followers. The former thought that oxygen activation was the most essential feature of respiration, while Wieland put H-activation in the fore. I could show that both processes were involved. I simply knocked out O_2 activation (and with it, respiration) by cyanide and then added methylene blue to the minced tissue. The dye restored respiration, replacing O_2 activation. It was reduced by activated H and then reoxidized spontaneously. During these experiments I became fascinated by the succino- and citrocodehydrogenase. These dehydrogenases differed from other dehydrogenases by being bound to structure, and "structure" had to mean something very important. They could not possibly be just ordinary metabolic enzymes, they had to have some general catalytic role. If this was so, then the whole of respiration had to be inhibited once the succino-dehydrogenase was inactivated, which could be done by malonic acid, as shown earlier by Quastel. So I added malonic acid to the minced tissue, and respiration stopped. This proved that succinic acid (and citric acid) had to have some general catalytic activity and could not be simply metabolites, as thought before. These ideas were later completed by Krebs and are the foundation of the so-called "Krebs cycle." It was partly this discovery of the C_4 dicarboxylic acid catalysis which was honored later by the Nobel prize.

I also became interested in vegetable respiration, being convinced that there is no basic difference between man and the grass he mows. Plants, at that time, were divided into two groups: the "catechol oxidase" and "peroxidase" plants. I started with the catechol oxidase plants which contain catechol and a strong catechol oxidase. I simplified the accepted, rather complex ideas about this oxidation system. Then I shifted to "peroxidase plants" which are called so because they contain peroxidase in high concentration. If peroxide is added to a mixture of peroxidase and benzidine, immediately an intense blue color appears due to the oxidation of benzidine. I found that if the reaction was performed with the plant juice, instead of purified peroxidase, there was a very short delay, of a second or so, in the benzidine reaction. This fascinated me. There had to be present a reducing agent which reduced the oxidized benzidine, the delay corresponding to the time necessary to oxidize away this unknown reducing agent, later to be known as ascorbic acid.

I mention this story in such detail because it illustrates the basic trait of my way of working. I make the wildest theories, connecting up the test tube reaction with broadest philosophical ideas, but spend most of my time in the laboratory, playing with living matter, keeping my eyes open, observing and pursuing the smallest detail. The current fashion is to avoid making theories (they may be wrong!) and limit one's observations to reading pointers. I think that an intimate finger-tip friendship with living matter is still important for the biologist. By working in this way, usually something crops up, some small discrepancy, which, if followed up, may lead to basic discoveries. The theories serve to satisfy the mind, prepare it for an "accident," and keep one going. I must admit that most of the new observations I made were based on wrong theories. My theories collapsed, but something was left afterwards.

I also made theories about the adrenal gland which led me to assume that the reducing agent of peroxidase plants should also be present in the adrenal cortex in high concentration. I found it was present (though the underlying theory turned out to be wrong later).

Hamburger's death made an end to all this. His successor was a psychologist who disliked chemistry and disliked me with it. I thought that I had to give up altogether, being still a beginner in science, who had no more money and no foreign diploma. So I sent my wife with our child back to Hungary to her parents and prepared for the end. I saw no chance left. For a farewell to science I went to attend the International Physiological Congress at Stockholm (1926). The presidential address was delivered by Sir Frederick Gowland Hopkins, who, to my surprise, mentioned my name three times, more than anyone else's. So, after his lecture I picked up all my courage and addressed him. "Why don't you come to Cambridge?" he asked. "I will see to it that you get a Rockefeller fellowship." And so he did. He was, and still is, a mystery to me. He was the man who had the most influence on my scientific development though I never talked to him about science and heard him speak but once or twice. His papers were not especially fascinating, yet he had a magic influence on the people around him. That little unassuming man, with all his childish vanity, was a humble searcher of truth. What his individuality proclaimed was that in spite of all the hard work involved, research is not a systematic occupation but an intuitive artistic vocation.

In Cambridge I isolated the reducing agent found at Groningen. I crystallized it from oranges, lemons, cabbages, and adrenal glands. I knew it was related to sugars, only did not know which. "Ignosco" meaning "don't know" and the ending "ose" meaning sugar, I called this carbohydrate "Ignose." Harden, the editor of the *Biochemical Journal,* did not like jokes and reprimanded me. "Godnose" was not more successful and so, following Harden's proposition, I called the new substance "hexuronic acid" since it had 6 C's and was acidic. I got my Ph.D. for it.

The trouble was that I could make it on bigger scale from one material only, adrenal glands, but these were not available in England in sufficient quantity. So I accepted N. Kendall's invitation to go to the Mayo Clinic, at Rochester, Minnesota, where ample material from the St. Paul slaughterhouses was available. I worked for one year in the USA, to return to Cambridge with 25 grams of "hexuronic acid" in my pocket, most of which I gave to Haworth, the great carbohydrate chemist, who undertook the constitution analysis.

Hungary, at that time (1932), had a very outstanding Minister (Secretary) of Education. He wanted to modernize Hungarian science and asked for my help. So I accepted the chair of medical chemistry at the University, Szeged, and left Cambridge with a heavy heart, for the University of Szeged. My laboratory was soon filled with able young researchers. I went back to oxidation and was soon fascinated by an unknown yellow dyestuff, "cytoflave," with its splendid fluorescence and reversible reducibility. Having no spectroscope, I could not describe it properly. Now it is called riboflavin. I also became interested in lactocodehydrogenase, found its activity linked to a coenzyme, a nucleotide, which I isolated in quantity in order to hydrolyze it for its analysis. I had a strong hunch that pyridine derivatives were involved as bases. I wanted to precipitate the hydrolysate with platinic chloride but when I came to it I found, to my dismay, the bottle of platinic chloride empty. With the shortage of chemicals my efforts to get hold of some platinic chloride failed, and so my hydrolysate just withered away. I followed practically the same route which led Warburg to the discovery of the pyridine nucleotides.

One day a nice young American-born Hungarian, J. Swirbely, came to Szeged to work with me. When I asked him what he knew he said he could find out whether a substance contained Vitamin C. I still had a gram or so of my hexuronic acid. I gave it to him to test for vitaminic activity. I told him that I expected he would find it identical with Vitamin C. I always had a strong hunch that this was so but never had tested it. I was not acquainted with animal tests in this field and the whole problem was, for me, too glamourous, and vitamins were, to my mind, theoretically uninteresting. "Vitamin" means that one has to eat it. What one has to eat is the first concern of the chef, not the scientist.

Anyway, Swirbely tested hexuronic acid. A full test took two months but after one month the result was evident: hexuronic acid was Vitamin C. We made no secret of this and finished the test which left no doubt about the identity. So, we (Haworth and I) rebaptized hexuronic acid to "ascorbic acid."

There we were. Ascorbic acid seemed medically most important but there was none of it, and none of the available vegetable sources allowed big-scale preparation. Adrenals were not available, in quantity, in Hungary. As it happened, Szeged is the center of the paprika (red pepper) industry. Paprika was not available at Cambridge. I once saw it on the market but the vendor cautioned me that it was poisonous. One night we had fresh red pepper for supper. I did not feel like eating it and thought of a way out. Suddenly it occurred to me that this was practically the only plant I had never tested. I took it to the laboratory and about midnight I knew that it was a treasure chest of vitamin C, containing 2 mg per gram. A few weeks later I had kilograms of crystalline Vitamin C which I distributed all over the world among researchers who wanted to work on it. This soon made complete analysis and synthesis possible. I received my Nobel prize partly for this work which also led to another unexpected discovery. When I still had only impure but highly concentrated solutions of ascorbic acid we tried my extracts in cases of Henochs' Purpura. In scurvy there is a great capillary fragility causing subcutaneous bleeding, so it seemed logical to try my extracts in purpura (subcutaneous bleeding). They worked. When I had crystalline ascorbic acid we tried it again, expecting a still stronger action. It did nothing. Evidently, my impure extract contained an additional substance responsible for the action. I guessed that it might be "flavones" which did the trick. My guess proved right. I isolated the flavones from "paprika" and they cured purpura. I called this group of substances Vitamin "P." I used the letter P because I was not quite sure that it was a vitamin. The alphabet was occupied only up to F so there was ample time to eliminate "P" without causing trouble if the vitamin nature became disproved.

I felt I had now enough experience for attacking some more complex biological process, which could lead me closer to the understanding of life. I chose muscle contraction. With its violent physical, chemical, and dimensional changes, muscle is an ideal material to study. If one embarks on such a new field one usually does not know where to begin. There is one thing one can always do, and this I did: repeat the work of old masters. I repeated what W. Kühne did a hundred years earlier. I extracted myosin with strong potassium chloride (KCl) and kept my eyes open. With my associate, I. Banga, we observed that if the extraction was prolonged, a more sticky extract was obtained without extracting much more protein. We soon found that this change was due to the appearance of a new protein "actin," isolated in a very elegant piece of work by my pupil, F. Straub, while I "crystallized" myosin. Myosin, evidently, was a contractile protein, but the trouble was that *in vitro* it would do nothing. A contractile protein should contract wherever it is. So we made threads of the highly viscous new complex of actin and myosin, "actomysin," and added boiled muscle juice. The threads contracted. To see them contract for the first time, was perhaps the most thrilling moment of my life. A little cookery soon showed that what made it contract was ATP and ions. My conclusion, that muscle contraction was essentially an interaction of actomyosin and ATP, was soon strongly attacked, so I developed (later at Woods Hole) the method of glycerination, and glycerinated (extracted with diluted glycerol at low temperature) the psoas muscle of the rabbit. This method is now widely used for conservation of biological material such as sperm. On addition of ATP, my glycerinated muscle contracted, developing the same tension as it developed maximally *in vivo*. This satisfied me and I was sure that in a few weeks' time the whole problem of muscle contraction would be cleared up, but ten years later I still did not understand muscle, which made me conclude that something had to be missing from our basic ideas, something that was essential for the understanding of energy transformation. So I left muscle to find what this something is. This took me, gradually, into my present field, that of electronic dimensions and mobility.

As a temporary president of my university at Szeged, I tried to put into action the ideas picked up in the west. I created an intense cultural life among students which culminated in our producing Hamlet, and producing it well. But my democratic ideas brought me more and more into conflict with the rising tide of fascism. It was not I who went into politics. Politics came into our lives and when books were burned and my Jewish friends were prosecuted I had to say "yes" or "no." I said "no" and when later, during World War II a group of leading Hungarians came, secretly, to me and asked me to do something to save Hungary from Germany's grip, I went, under cover of an alleged lecture, to Istanbul to get in touch with the British and American diplomats to see what could be done. This was a risky undertaking, for German-occupied territory had to be crossed and Istanbul was the spying center, with highly developed techniques, and I was a newcomer in this business. I felt that I could be more useful if I did not go merely as a private individual to Istanbul and took a chance. I went to our Prime Minister, Mr. M. Kallay, and told him about my plans. Outwardly, Mr. Kallay was a Nazi, but I suspected that he was a good Hungarian, waiting for his chance to bring his country over to the other side. My guess was right. Instead of having me arrested he asked me to represent him and convey certain messages to the Allies. In Istanbul I succeeded in getting in touch with the head of the British Secret Service, making with him detailed plans which soon had the blessing of London. What made these dealings exciting was that, till the end, I could not know for certain whether I was dealing with the British, or the German Secret Service. This I could only find out later, when crossing German territory. Not being arrested on my return, I was finally sure that it was the British to whom I had talked.

Unfortunately, the secret of my mission leaked out, and I could not set up a secret wireless station which was essential for my plans. I was placed under house arrest. Hitler demanded my delivery. Later, when he occupied Hungary, I avoided final arrest by the Gestapo only by an inch, owing my escape more to good luck than ability. Arrest would have meant a very painful death. Even my daughter had to go into hiding, an arrest warrant having been issued also against her. Working against Hitler and living underground was full of colors which were not always pleasant. I expected to be killed so I wrote up my observations on muscle, which I did not want to be lost. I sent them for publication in the *Acta Scandinavica* to my friend Hugo Theorell. Not knowing where I was, he corroborated acceptance by wire, "care of Swedish Legation, Budapest." Fate would have it that at that time I was actually hiding at the Swedish Legation, and so Theorell's wire gave me away. The Gestapo immediately searched the surrounding houses for subterranean exits from the Swedish Legation, which served as a warning. Also, a hint from a friendly German diplomat made it evident that arrest was imminent. So Per Anger, the head of the Legation, smuggled me out in the back of his car the next night. Shortly after, the Nazis broke into the Swedish Legation, searched, robbed, and practically destroyed it. Then followed a series of exciting situations shared by my wife. At the end, we had to part, hiding together becoming too risky. Two of my hiding places were destroyed by bombs shortly after I left them, and, in the end I could avoid arrest only by hiding in the vicinity of the Soviet lines where the Gestapo did not dare to come.

The profound disgust we felt for Nazism made us guilty of a fatal sin in politics—wishful thinking. It made us believe that after Hitler was finished all we had to do to bring on the great golden age of peace was to show good will towards the Soviets. It is true that in the short communist period of Hungary, after World War I, the Communists behaved very badly, but that was long ago. A new world was to come. This was a most tragic error with fatal consequences. From my hiding places I contacted Governor Horthy, who was still the master of the situation. We met in secret, and I offered my services as an envoy to the Allies to prepare Hungary's joining them. He seemed to accept but when he noticed my friendly disposition towards the Soviets he edged out of the room and I never saw him again. I can reproach only myself for this failure. I should have taken Horthy's mentality into account. He hated Russia and feared it.

Personally, I did not expect a better treatment from the Soviets than I had expected from Hitler, having given my heavy golden Nobel Medal to Finland when the Soviets declared war on her, and this medal meant more than just gold. So I was not surprised when, after the "liberation" of Budapest, a Soviet patrol, with an English-speaking major at its head, came searching for me. I gave myself up. To my surprise the patrol did not come to arrest me but to bring me to safety on Molotov's personal order. I refused to go along, not wanting to leave my wife's big family in the very dangerous situation then prevailing in the Capital. So the whole family was taken to safety, while my wife and I were taken to Malinowski's headquarters where we were fed back to life with utmost care and consideration. Later, I was invited to Moscow where I spent two months and attended the Centennial Celebration of the Academy, finishing up with a trip to Armenia.

I went to Moscow with the hope of seeing Stalin. What made me want to meet him was the fact that the Soviet Army in Hungary behaved very badly. Near my home town a Hungarian regiment laid down its arms, not wanting to fight for Hitler. The whole regiment was crowded into a small prison where it was soon exterminated by typhus fever. In Budapest the ends of streets were suddenly closed by Soviet soldiers and all the younger men were herded together. Their documents were taken away, which wiped out their identity. About 30–40,000 men were arrested this way and then herded to Czegled, a nearby camp where there was no food and poor sanitation. Dysentery and typhoid began to decimate them. The screams could be heard from long distances. Those who were left were herded into trains, the doors of which were sealed; nobody knew where they went. We could not guess, at that time, that these people were simply taken to Russia as slaves, the whole transaction recalling the darkest days of African slave trade. With our wishful thinking we tried to find excuses for the Soviet atrocities. We even tried to find excuses for the individual misbehavior of Soviet soldiers; war is a beastly business, and makes beasts of men. So, I went to Moscow with the hope of being able to tell Stalin what was going on in Hungary, that we Hungarians wanted to be friends with the Soviet but couldn't be if he did not end this rule. I asked for an interview and was taken into the Foreign Office before Mr. Decanozov, who had to find out what I wanted from Stalin. Mr. Decanozov must have been a very high official because he was later executed together with Beria. He asked me what I wanted. I told him. His reaction was unexpected: he began to shout. At this moment I felt that what I thought to be the overzeal of local commanders was all planned in Moscow. Going home, I still continued working for an understanding with the Soviets. If we had to live together, we had better understand each other. The Russian people are a fine people whom one cannot help liking once one knows them.

I thought, also, to have another vocation: to help rebuild the devastated culture of Hungary and save our leading intellectuals from starvation. I could help only a limited number, so I started a new "Scientific Academy," and selected its members, 50 or 60. The Academy consisted, chiefly, of a grocery store which was kept well-stocked and from which members of the Academy could take what they needed, free of charge. A friend of mine helped me finance this enterprise. He also helped me to establish a new school of biochemical research. This was not easy, because, to find a potato in those days was a full-time job, and if I wanted my associates to work I had to feed them. My laboratory looked like a chick embryo with its great vitelline sack. It consisted of a big kitchen, led by my wife, and a laboratory, led by myself.

Personally, I had no complaints against the Soviet, who always gave me the most distinguished treatment. In order to stock my "Academy" I needed trucks and with my friend, the writer, L. Zilahy, we asked for trucks from Marshall Voroshilov who readily complied. With these trucks we established a travel agency. In those days everybody wanted to get away from the capital but there was no transportation. So, we could charge high prices for taking people to the country, where, with the fares collected, we bought food for the Academy.

In spite of the personal favours it became more and more difficult for me to find excuses for the Soviet's behaviour, which I still did not understand. One day I went to Switzerland to restore my health on my skis. The Soviet Commander used my absence to get rid of my capitalist friend who financed my laboratory and Academy. He was kidnapped. The next day the Communist papers brought out articles about him, saying that he stole money and ran away. I was informed about the real happenings and put in all my influence to have my friend released. The authorities miscalculated. If I had been in Hungary I could have done nothing. At large, in Switzerland, I could call the World's attention to what was going on in Hungary, and this would have been a bad point in Moscow for our local Communist leaders. My wires to the Prime Minister and the Communist Dictator left no doubt about my determination. My friend was released, and given a passport to leave the country, having "seen too much." To prevent a second kidnapping, my wife accompanied him by car to Switzerland, hoping that the Soviet would not risk touching her. In Switzerland I learned from my friend what was going on behind the prison walls, which I could never find out at home. This filled me with such profound disgust that I was unable to return. To go back and resist the Soviet made no sense and accepting favours was impossible. So I decided not to return. Eventually, I found my haven in Woods Hole, where I am enabled, now, by American generosity, to work unhampered by any other factor than my own personal limitations.

* * *

The sole general interest in this story is that it sheds a vivid light on the turbulence of our days, showing the conflict between my scientific world and prescientific surroundings which were immiscible. Looking back gives me the feeling of frustration. Resisting Hitler, building academies, research schools, living for years with a finger on the trigger instead of fingering test tubes—and all this to see the part of the world I worked for trodden down as a colony, and to see mankind on the brink of extinction. The idea of being killed for my ideas never frightened me. At one time it even seemed natural. But to have spent so much life and energy in vain is depressing, and I have to ask myself, as so many other scientists must do: has research any sense? Should science not be stopped till man reaches the maturity necessary to deal with the forces which science creates, without the danger of self-destruction?

In a way, the question has no sense, for scientific progress cannot be stopped. Human curiosity cannot be quenched. The question is, rather: does scientific progress offer a way out? To this question my answer is an emphatic "yes."

In the preamble, I have touched upon two facets of science, its ways of thinking and the tools it creates. The danger of our days is that politics has run away with the tools, leaving the way of thinking behind. The forces created by science can be handled only by the mentality which created them. So if there is a way out it is not in suppressing, but in spreading science till scientific thought becomes sufficiently strong to create its own world order.

It may be objected that human relations are not dominated by thoughts but by morals, and science has no moral content. Morals are the simple prescriptions which make living together possible. They have no intrinsic meaning. It would make little sense to say to a tiger: "Thou shalt not kill," or preach to a mouse: "Thou shalt not steal." But a human society cannot exist without such rules.

But is it true that science has no moral content? Is science not more than just a method of thinking, tools, or a collection of data and books? Is science not a living society? I think it is. To me, science, in the first place, is a society of men, which knows no limits in time and space. I am living in such a community, in which Lavoisier and Newton are my daily companions; an Indian or Chinese scientist is closer to me than my own milkman. The basic moral rule of this society is simple: mutual respect, intellectual honesty, and good will. So I think science does have its moral code which it offers as its third facet on which a new world order can be built. Science has raised man from stench and dirt, liberated him from the miasmas which decimated him in earlier times. It allows the bearing of children without fear. It has already shown the possibility of a dignified life, the expectation of which it has greatly extended. It is true, it has reduced man to a very modest place in Creation, but, then, why not try to lift ourselves, accepting the responsibility for our own fate? Why pull down one another, further poisoning our own atmosphere, showing how easily life can be wiped out? Science has opened endless possibilities for expansion if we work together instead of snatching small advantages from one another. Science has helped us to understand and master Nature. Maybe it will help us to understand and master ourselves, creating an elevated new form of human life, the wealth and beauty of which cannot be pictured today by the keenest imagination.

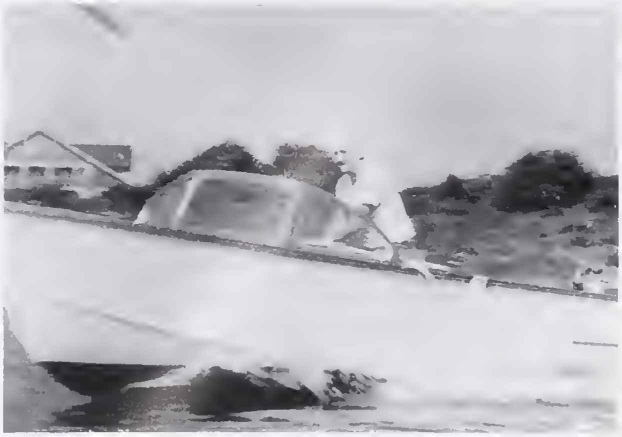
Jesting after a donkey ride in Hungary.

Courting joyously.

Masquerading with Marta ("Profne") at Seven Winds.

Horseback riding.

Fishing off the Gut in Woods Hole. "It's more exciting not to catch a big fish than not to catch a little one," he once said. But he caught some big fish both in the sea and in the laboratory.



Racing in his 15-foot outboard motorboat.

Lecturing at the Cold Spring Harbor symposium on muscle (1972).

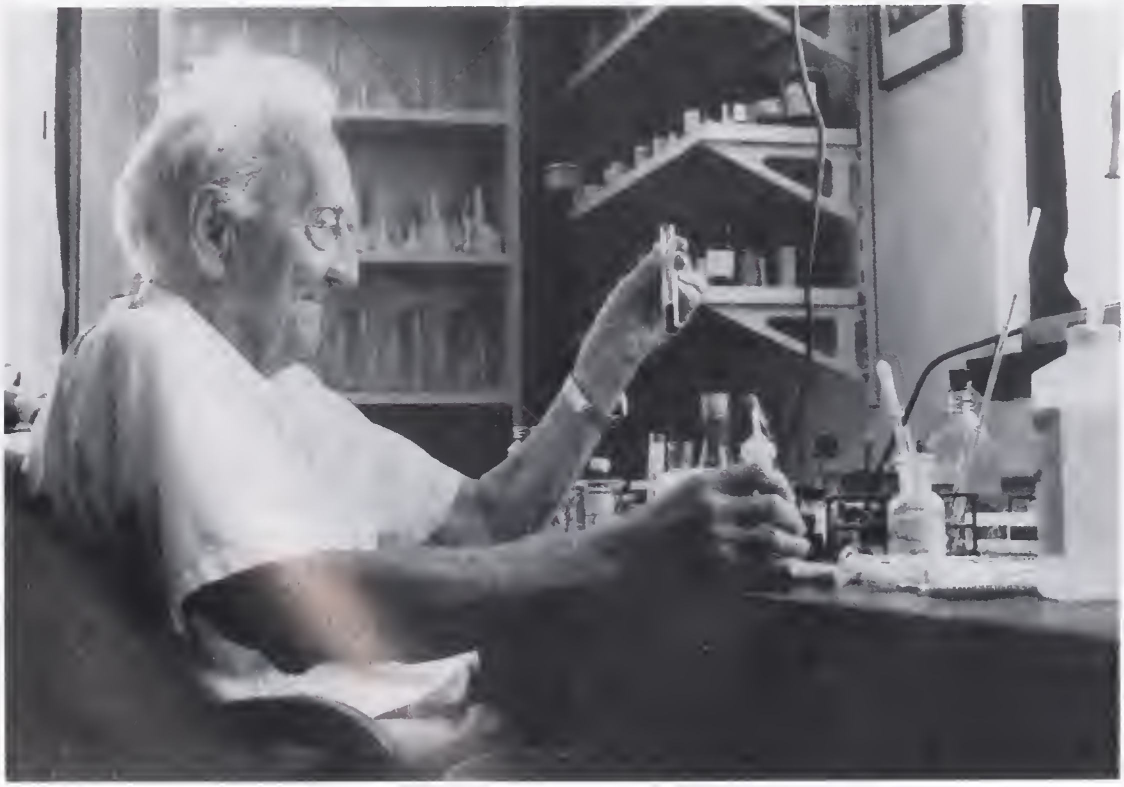
Lecturing at the symposium "Search and Discovery" held in his honor at the Boston University Medical School (1975).

Listening at the "Search and Discovery" symposium. (With Benjamin Kaminer.)

Chatting with Andrew Szent-Györgyi. (Also with Irvin Isenberg and Eva Szent-Györg

Motorbiking to his laboratory in Woods Hole.

Climbing in the laboratory to get chemicals.



Seeing the unseeable.

Checking what he's seen in the spectrophotometer. (With Jane McLaughlin.)

Leading the parade in Woods Hole on the Fourth of July.

Toward a Molecular Understanding of Cholesterol and Atherosclerosis

Joseph L. Goldstein and
Michael S. Brown

University of Texas Health Science Center

The most striking biochemical abnormality in human atherosclerosis is the massive accumulation and deposition of cholesteryl esters in the artery wall. This cholesteryl ester is derived primarily from a cholesterol-carrying protein called low density lipoprotein (LDL) that circulates in the bloodstream. The lipoprotein enters the artery wall and deposits its cholesterol in places where the cells that line the wall (the endothelium) have been injured. The rate at which LDL deposits its cholesterol in arteries is influenced by several so-called "risk factors" that have been defined by epidemiologic studies. Many of these risk factors damage the endothelium and thereby promote the penetration of plasma LDL into the wall. These include cigarette smoking, hypertension, diabetes mellitus, and poorly understood genetic influences that determine the vulnerability of an individual's vessels to cholesterol deposition. The more LDL in blood, the faster the development of atherosclerosis. Conversely, if the level of LDL is low enough, atherosclerosis is slow to develop, even in the face of all of the other risk factors.

Epidemiologic studies have revealed the surprising fact that more than half of all people in Western industrialized societies, including the United States, have levels of circulating LDL that are above the threshold at which atherosclerosis is accelerated. In the past these concentrations of LDL have been considered "normal" in the sense that they are the usual values found in such populations. However, they seem not to be normal for the human species in the sense that they lead to accelerated atherosclerosis.

What determines the concentration of LDL in the bloodstream? Why do half of all Americans have concentrations of LDL that place them at high risk for developing atherosclerosis? Answers are emerging from studies of a class of proteins, called *lipoprotein receptors*, that were discovered eleven years ago. Projecting outward from the surfaces of cells in the liver and other organs, these receptors bind circulating LDL. Binding initiates a process by which the LDL is taken up by the cells and degraded, yielding its cholesterol for cellular use. LDL receptors thus perform a dual function: they supply cholesterol to cells and they remove LDL from the bloodstream.

The cells of the body produce varying numbers of LDL receptors, depending on their needs for cholesterol. When the cells' needs are high, they produce large numbers of receptors, LDL is removed rapidly from the circulation, and the level of LDL in blood is kept below the threshold for rapid development of atherosclerosis. On the other hand, when cells accumulate excess cholesterol, they produce fewer LDL receptors and take up LDL at a reduced rate. This protects the cells against ex-

Michael S. Brown

Michael S. Brown was born in New York City in 1941. He received his bachelor's degree from the University of Pennsylvania in 1962 and his M.D. from the University's School of Medicine in 1966. Following his internship and residency training, Brown became an Assistant Professor at the University of Texas Health Science Center at Dallas in 1971. Within five years he was promoted to a full Professor of Medicine. In 1977 he was appointed the Paul J. Thomas Professor of Genetics and Director of the Center for Genetic Disease at the University of Texas Health Science Center in Dallas, a position he continues to hold. He is a member of the U. S. National Academy of Sciences and the American Academy of Arts and Sciences. Brown and his colleague, Dr. Joseph L. Goldstein, have shared a number of awards for their discovery of receptors that control the level of cholesterol in blood, including the Louisa Gross Horwitz Award (1984) and the Nobel Prize in Physiology or Medicine (1985).

Joseph L. Goldstein

Joseph L. Goldstein was born in Sumter, South Carolina, in 1940. He received his B.S. degree from Washington and Lee University in 1962, and his M.D. degree from the University of Texas Southwestern Medical School, Dallas, in 1966. Following his internship and residency, he became an Assistant Professor of Medicine at the University of Texas Southwestern Medical School and Head of the Division of Medical Genetics. By 1976 he had been appointed Professor of Internal Medicine. In 1977 he was appointed Chairman of the new Department of Molecular Genetics at the University of Texas Health Science Center, Dallas, and the Paul J. Thomas Professor of Medicine and Genetics. Goldstein is a member of the National Academy of Sciences, and the American Academy of Arts and Sciences, among others. Together with Dr. Michael S. Brown, Goldstein has received many awards for their discovery of receptors that control cholesterol metabolism, including the Louisa Gross Horwitz Award (1984) and the Nobel Prize in Physiology or Medicine (1985).

cess cholesterol accumulation, but at a very high price: the reduction in the number of receptors decreases the rate at which LDL is removed from the circulation, the level of LDL rises, and atherosclerosis is accelerated.

Accumulating evidence has led us to propose that the high level of LDL in many Americans is attributable to a combination of factors that lead to a diminished production of LDL receptors. One of these factors is a general tendency of humans to produce a relatively small number of LDL receptors, as compared with animals of other species. This tendency is aggravated by a diet that is rich in cholesterol and saturated fats derived from meat and dairy products. Such a diet causes cholesterol to accumulate in cells of the liver, leading the cells to further decrease their production of LDL receptors. Much of our insight into the consequences of diminished LDL receptor production has come from studies of familial hypercholesterolemia (FH), a human disease in which the receptors are diminished not as a result of a dietary excess, but as a result of a defect in the gene encoding the receptors. These studies have led to the suggestion that LDL receptor deficiency, aggravated by a high fat diet, is a major cause of high blood cholesterol levels and atherosclerosis in the general population of the United States.

Recent insights into the function of the LDL receptor suggest a new approach to the treatment of hypercholesterolemia. This therapeutic approach is based on the concept of receptor regulation. FH heterozygotes have one normal gene turning out functional receptors and one faulty gene turning out nonfunctional receptors. Experiments in animals and in human beings show that it is possible, through the use of diets and drugs, to stimulate the normal receptor gene to produce twice the normal number of functional LDL receptors, which in turn lowers the plasma LDL level by as much as 50%. The most effective drug regimen involves the combined use of a bile acid binding resin (such as cholestyramine or colestipol) and an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (such as mevinolin or compactin).

Inositol Lipids and Cell Signalling

Michael J. Berridge

Unit of Insect Neurophysiology and Pharmacology, Department of Zoology, Cambridge, U. K.

Receptors that act by stimulating inositol lipid hydrolysis generate two intracellular signalling pathways. The water-soluble inositol 1,4,5-trisphosphate ($Ins1,4,5P_3$) released to the cytosol mobilizes intracellular calcium. $Ins1,4,5P_3$ is metabolized via two pathways, either by dephosphorylation ($Ins1,4,5P_3$ to $Ins1,4P_2$ to $Ins4P$ to inositol) or by a phosphorylation/dephosphorylation pathway ($Ins1,4,5P_3$ to $Ins1,3,4,5P_4$ to $Ins1,3,4P_3$ to $Ins3,4,P_2$ to $Ins3P$ to inositol). The significance of the latter pathway is that $Ins1,3,4,5P_4$ might function as a messenger to control the entry of external calcium. In addition to these agonist-sensitive pathways, there are agonist-insensitive pathways responsible for the formation of $InsP_5$ and $InsP_6$ which are attracting great interest—they may function as neurotransmitters. The diacylglycerol that remains within the membrane activates protein kinase C to initiate a variety of modulatory processes including inhibition of inositol lipid hydrolysis.

Such feedback interactions between second messenger pathways may account for the oscillations in calcium recorded from many different cells. There are two types of calcium oscillation: (1) a pseudo-sinusoidal type apparently the result of a cytoplasmic oscillator located in the endoplasmic reticulum, and (2) a type where periodic bursts of intracellular calcium occur under control of receptors at the plasma membrane. $Ins1,4,5P_3$ is thought to play a role in both the receptor-controlled and cytoplasmic oscillatory mechanisms which may co-exist in many cells. Such oscillatory activity might form the basis of a novel frequency-dependent intracellular signalling system for controlling a variety of cellular functions.

This ubiquitous signalling system functions throughout a cell's life history. After controlling early events during maturation, fertilization, and cell proliferation, it is redeployed in differentiated cells to control a host of cellular processes including metabolism, contraction, and secretion. A particularly important role is found in the nervous system where inositol lipid hydrolysis mediates the action of many neuromodulators.

Michael J. Berridge

Michael John Berridge was born in 1938 in Gatooma, Rhodesia. He received his B.S. from The University College of Rhodesia and Nyasaland in 1960 and his Ph.D. from the University of Cambridge in 1964 under Sir Vincent Wigglesworth. In 1969 Berridge accepted an appointment in the AFRC Unit of Insect Neurophysiology and Pharmacology at Cambridge where he is currently Deputy Director of [illegible]. He became a Fellow of Trinity College in [illegible] and was elected a Fellow of the Royal Society in 198[illegible]. He is best known for his discovery of the second messenger inositol triphosphate (IP_3) which plays a universal role in regulating most cellular processes including cell growth and synaptic transmission in the nervous system. He has received numerous awards and prizes for his work, including The King Faisal International Prize in Science and the Louis Jeantet Prize in Medicine.

How Proteins Move From the Cytoplasm Into Mitochondria

Gottfried Schatz

Biocenter, University of Basel, Switzerland

Each mitochondrion contains hundreds of different polypeptides. Virtually all of these are encoded by nuclear genes, synthesized in the cytoplasm, and subsequently imported to one of the four major intramitochondrial compartments: matrix, inner membrane, intermembrane space, and outer membrane. This process, which involves between 5–20% of the total proteins in a typical eukaryotic cell, is still incompletely understood. However, we are beginning to glimpse some details of how proteins recognize mitochondria and at which sites they enter these organelles.

In virtually all cases studied so far, the signal for directing a protein into mitochondria is located in a relatively short amino-terminal stretch of the polypeptide. With proteins synthesized as larger precursors, this targeting sequence usually occupies the amino-terminal tip of the presequence. If this targeting sequence is deleted, the protein is no longer imported; if the sequence is grafted onto a non-mitochondrial protein, then that protein is usually imported into mitochondria. In the absence of additional signals, these targeting sequences direct attached proteins to the mitochondrial matrix: they are "matrix-targeting" sequences. Matrix-targeting sequences do not share sequence homologies, but can form positively charged amphiphilic structures. Even completely synthetic presequences constructed from only a few types of amino acids function as matrix-targeting sequences as long as they fulfill the criteria mentioned above. Mitochondrial presequences can thus easily arise by DNA rearrangements or point mutations, which could explain how these sequences arose during the evolution of mitochondria from endosymbionts.

Proteins can enter mitochondria posttranslationally. This process can be duplicated *in vitro* using isolated mitochondria and purified precursor proteins. We have used an artificial precursor protein (a mitochondrial presequence fused to mouse dihydrofolate reductase) to study the conformational changes that accompany uptake of the protein into mitochondria. CD- and fluorescence measurements showed that the dihydrofolate reductase (DHFR) moiety in the fusion protein is folded nearly as tightly as that of the authentic mouse enzyme. Upon binding to energized mitochondria, the presequence inserts into the mitochondrial membranes and the DHFR moiety spontaneously unfolds on the mitochondrial surface. This reaction appears to involve acidic phospholipid head-groups of the outer membrane. Upon addition of ATP, the unfolded, surface-bound intermediate is chased into mitochondria in the absence of a membrane potential and refolds spontaneously in the matrix. Unfolding of the protein before translocation is the major rate limiting step of import, at least with isolated mitochondria.

To identify components of the translocation machinery we have isolated temperature-sensitive mutants defective in protein uptake into mitochondria. These mutants have lead to the isolation of two nuclear genes involved in the overall translocation process. One of these genes is a regulatory subunit of the processing protease in the matrix. We have also identified a 45 kD outer membrane protein that is essential for translocation to occur. Translocation occurs at sites where the two mitochondrial membranes are in close contact. We have purified these sites and are studying their protein components.

Gottfried Schatz

Gottfried Schatz was born August 18, 1936, near the Hungarian border in a little Austrian village that's population consisted of approximately two hundred peasants and more than one thousand geese. The sounds of his childhood (besides the chatter of geese) were largely Hungarian — Szent-Györgyi's language. His parents soon moved to the city of Graz where he began studying chemistry. There he became interested in biochemistry, but learned that this subject was not taught at his university. He then wrote to several noted biochemists, asking them for their latest reprints. David Green was particularly generous and sent him a large package with all of his reprints, a gesture that influenced him to become a mitochondriologist. He was a postdoctoral fellow with Efraim Racker in New York City from 1964 to 1966. After a brief return to Vienna, he joined the Biochemistry Department at Cornell University where he remained from 1968 to 1974. In 1974 he moved to Basel where he has been working ever since. Schatz is a member of the German Academy of Sciences "Leopoldina," and has received a number of awards during his career, including the Carlsberg Prize in 1982, the Hans Krebs Medal in 1986, and the Otto Warburg Medal in 1988.

"Zinc-Fingers"—A Novel and Ubiquitous Protein Motif For Nucleic Acid Recognition

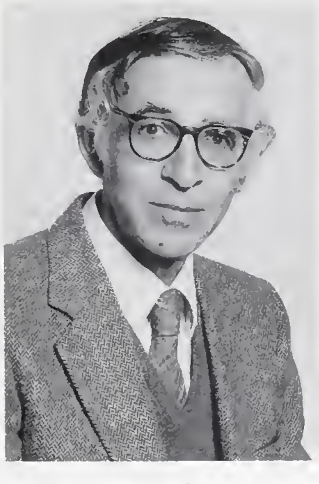
Aaron Klug

MRC Laboratory of Molecular Biology, Cambridge

Activation of the gene for 5S RNA in *Xenopus* and other eukaryotes requires the binding of a regulatory protein, called Transcription Factor IIIA (TFIIIA). We have purified this protein, and have shown that it has a remarkable repeating structure. Each structural unit or "mini-domain" consists of a small loop of about 30 amino acids folded around a zinc ion. The units have a relatively high proportion of conserved amino acids, including four invariant residues ligated to the zinc ion, which can form a common structural framework for chemically distinct domains. Each unit can therefore recognize a different short stretch of DNA. We have called these domains "Zn fingers," and have further shown that each finger binds to, and thereby recognizes the sequence on, about half a turn of the DNA double helix.

As we predicted, these nucleic acid binding fingers have turned up in many other regulatory proteins. It thus appears that TFIIIA is the type of a novel class of proteins whose modular design offers a large number of combinatorial possibilities for the specific recognition of many different nucleic acid sequences.

Two subclasses appear to have emerged, according to whether the zinc ion is ligated to a pair of cysteines and a pair of histidines or else to two pairs of cysteines. It remains to be seen whether this type of fold occurs in other proteins not involved in nucleic acid recognition.



Aaron Klug

Aaron Klug was born in 1926, educated at the Universities of Witwatersrand and Cape Town, and was an 1851 Exhibition Overseas Scholar in the Cavendish Laboratory, Cambridge (1949–1952). He was research Fellow at Birkbeck College (1953–1961) where he worked with Rosalind Franklin (until her death in 1958) and thereafter led a team working on virus structure. Since 1962 he has been a member of staff at the Medical Research Council Laboratory of Molecular Biology, Cambridge, (Joint Head of the Division of Structural Studies, 1978–1986, Director since 1986) and a Fellow of Peterhouse where he was Director of Studies in Natural Sciences until 1985. His work has been on the elucidation of the structure of large biological molecules and assemblies by X-ray diffraction and electron microscopy and the development of new methods for their study. His current research is on the structure of chromatin and a novel class of transcription factors. Elected F.R.S. 1969; Copley Medal, 1985; Foreign Associate of the National Academy of Sciences, U. S. A., 1984; Heineken Prize of the Royal Netherlands Academy of Science, 1979; Louisa Gross Horwitz Prize of Columbia University, 1981; Nobel Prize for Chemistry, 1982.

Finding Out How Actin and Myosin Produce Movement

Hugh Huxley

Brandeis University

Albert Szent-Györgyi and his colleagues made two fundamental discoveries in the early 1940's that laid the basis for all subsequent work on the mechanism of muscle contraction. They found that the protein then known as myosin, and long recognized as a major structural component of muscle, was in fact a complex of two proteins. One of these, which had the ATPase activity discovered a few years previously by Engelhardt and Ljubimova, was continued to be called myosin. The other component was named actin.

Hugh Huxley

Hugh Huxley was born on February 25, 1924 in Birkenhead, Chesire, England. He was originally trained as a physicist at Cambridge University, but joined a small molecular biology group there headed by Max Perutz and John Kendrew to begin (in 1948) his PhD work. After some initial studies on crystalline proteins, he discovered that live muscles would give quite good low angle X-ray diffraction patterns, showing a double hexagonal filament array and constant axial periodicities. Studies of the behavior and implications of these reflections comprised his PhD thesis. Following up this work, Huxley came to MIT as a postdoc to learn electron microscopy in Frank Schmitt's group, and it was there that he collaborated with the late Jean Hanson to put forward the overlapping interdigitizing filament model of striated muscle in 1953 and the sliding filament hypothesis in 1954. After returning to England, Huxley continued structural work on muscle, viruses, and ribosomes, first in Cambridge and then at University College London. He returned to Cambridge in 1962 when the Medical Research Council's Molecular Biology Laboratory opened, and worked there until 1987; during the last 10 years he was Deputy Director and Joint Head (with Aaron Klug) of the Structural Studies Division. During this time he made extensive use of the negative staining techniques on preparations of muscle and muscle proteins, particularly in studies of the assembly and polarity of muscle filaments. Subsequently, he developed improved X-ray diffraction techniques to study contracting muscle and made extensive use of synchrotron radiation in time-resolved studies of the process. Recently, Huxley moved to the United States to join the Rosenstiel Center at Brandeis University.

Huxley is a Fellow of The Royal Society and a Foreign Associate of the National Academy of Science. Huxley has received many distinguished honors for his work, including the Royal Medal of The Royal Society (London), the Louisa Gross Horowitz Prize, the E. B. Wilson Medal of the American Society of Cell Biology, and the Albert Einstein World Award of Science.

The second discovery was that artificial threads of the complex formed by actin and myosin (actomyosin) would contract when immersed in a solution of ATP and suitable ions. Subsequently (in the late 1940's), Szent-Györgyi was responsible for the introduction of the glycerinated muscle fiber preparation, which has been of great value in muscle research.

Since then the main focus of muscle research has been to understand the structural and biochemical mechanisms that enable actin and myosin to interact and convert chemical energy from ATP into mechanical work. There have been a series of major advances in our knowledge of the system, often closely linked to significant advances in a variety of experimental techniques, and a remarkable realization of the extent to which closely similar proteins are involved in many motile phenomena in non-muscle cells. However, although the main outlines and many of the details of how myosin and actin assemble and interact have been established, certain very basic and interesting aspects of the mechanism still remain tantalizingly just beyond the range of current techniques and provide a fascinating challenge for future work.

The Future in Biological Research: What is Possible and What is Ethical

Daniel E. Koshland Jr.

University of California, Berkeley

Predictions of the future are always cloudy. In the 1800's the Commissioner of Patents planned for the ending of the Patent Office, since he had deduced that, at the contemporary rate of discovery, all possible inventions would be made in a few years. Today, the potentialities for biological research seem almost limitless. The conquest of disease seems possible at the scientific level, even though it remains difficult at the economic level. Some of the applications of biological research which are clearly possible can reorient social relationships in ways that some regard as unethical. There may indeed be a widening gap between what scientists could do and what they are allowed to do. Some of the future potentialities of biological research, and some of the ethical concerns which they may engender, will be discussed.

Daniel E. Koshland Jr.

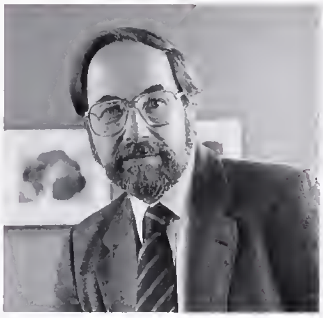
Daniel E. Koshland Jr. was born on March 30, 1920 in New York City. He received his B.S. from the University of California, Berkeley, and his Ph.D. from the University of Chicago. After a postdoctoral period at Harvard, he had joint appointments at Brookhaven National Laboratory and the Rockefeller University. Since 1965 he has served as Professor in the Department of Biochemistry at the University of California, Berkeley. He is a member of the National Academy of Science, and has received many honors for his achievements, including the T. Duckett Jones Award of the Helen Hay Whitney Foundation, the Edgar Fahs Smith Award of the American Chemical Society, and the Rosensteil Award of Brandeis University. Koshland is widely known for his studies on enzyme mechanisms and the control of enzyme action, and more recently for his studies on sensory systems.

The Molecular Basis of Differential Gene Transcription

David Baltimore

Whitehead Institute, Massachusetts Institute of Technology

The antibody light chain gene is only transcribed in more mature B-lymphocytes. A protein factor, NF-κB, binding to the DNA of the gene's enhancer region appears centrally responsible for this exquisite cell specificity of transcription. The factor exists in early preB-lymphocytes, and other cells, as an inactive precursor in the cytoplasm. Activation of such cells with a mitogen or an active phorbolester causes the precursor to gain DNA binding activity, probably by release of an inhibitor, and to rapidly migrate to the nucleus. Further studies on this model differentiation system will be described. The long-term goal is to understand a discrete step of mammalian cellular differentiation.



David Baltimore

David Baltimore was born on March 7, 1938 in New York City. He received his B.A. with honors in chemistry from Swarthmore College in 1969, and his Ph.D. in biology from the Rockefeller University in 1964. In 1968 Baltimore served as an Associate Professor at MIT and became Professor of Biology there in 1972. He was appointed American Cancer Society Research Professor in 1973, and the following year he joined the staff of the MIT Center for Cancer Research, where he remained until he was named Director of the Whitehead Institute in 1982. He is a member of the National Academy of Science, the American Academy of Arts and Sciences, and the Pontifical Academy of Sciences. In 1975 Baltimore received the Nobel Prize in Physiology or Medicine for his discovery of reverse transcriptase and the interaction between tumor viruses and the genetic material of the cell. His current research covers three areas: cancer-inducing viruses, the immune system, and poliovirus. In each field, studies are defining the biochemical events underlying gene expression and gene structure in the mammalian cell.

The Cancer Problem: An *In Vivo* Approach

Philip Leder

Harvard Medical School

Cancer is not a genetic disease in the conventional sense, but it is a genetic disease in that it reflects the fundamental disturbance of one or more genes in a somatic cell. During the past decade a number of normal genes have been identified that can undergo mutation and, in their altered form, contribute to the development of malignancy. Many of these oncogenes, in their normal forms, are expected to be involved in the control of cell growth. Our work over the past few years has evolved from an exploration of an oncogene that is involved in the human malignancy known as Burkitt lymphoma. In this disorder, one of the growth controlling genes, c-MYC, has been moved from its normal chromosomal position to a new one; a movement that results in its activation as an oncogene. Our exploration of this system led us to develop a hypothesis regarding the activation of this oncogene which involved the disruption of its regulation. We have tested that notion and other aspects of the mechanism of oncogenesis using transgenic mice in which an activated oncogene is artificially inserted into the animal's germline and is then passed on to its progeny. Using this system, we have tested the multi-hit hypothesis of carcinogenesis, the extent to which certain oncogenes can induce malignancy in a variety of cellular backgrounds and the extent to which these oncogenes require the complementary action of other oncogenes in order to induce malignancy.

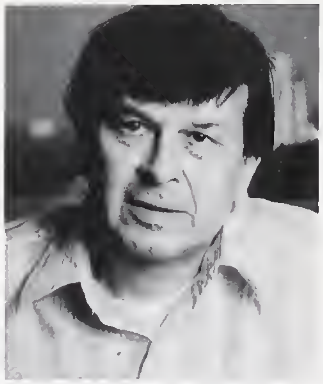
Philip Leder

Philip Leder was born on November 19, 1934 in Washington, DC. He is the John Emory Andrus Professor and Chairman of the Department of Genetics at the Harvard Medical School and Senior Investigator of the Howard Hughes Medical Institute. Leder, a graduate of Harvard College and Medical School, has carried out research in molecular genetics for twenty-five years during which time a revolutionary growth in our knowledge of the operation of genes has occurred. His current work applies the tools of the new genetics to the cancer problem in an effort to understand the genetic basis of malignant disease. Leder has received many honors and awards during his career, including the Bristol Meyer Award in 1985 and the Albert Lasker Medical Research Award in 1987.

Transmissible and Non-Transmissible Dementias as Brain Amyloidoses

Carleton Gajdusek

NINCDS/National Institutes of Health



Carleton Gajdusek

Carleton Gajdusek was born in 1923 in Yonkers, New York. As an undergraduate he attended the Embryology Course at the Marine Biological Laboratory. He received his B.S. in biophysics from the University of Rochester in 1943 and his M.D. from Harvard Medical School in 1946. After an internship and residencies in pediatrics, Gajdusek was a Research Fellow with Linus Pauling, Max Delbruck, and John F. Enders. His research interests focus on child growth, development, and behavior, and disease patterns in primitive cultures throughout the world. He investigated virus infections and immunological disorders, with a particular interest in slow-acting viruses. Currently, Gajdusek is studying degenerative central nervous diseases as Chief of the Laboratory of Central Nervous System Studies, NINCDS, National Institutes of Health, a post he has held since 1970. He is a member of the National Academy of Science and many other distinguished societies world-wide. Gajdusek has received numerous honors for his achievements, including the Nobel Prize in Physiology or Medicine in 1976.

Reference: *Biol. Bull.* **174:** 214–233. (June, 1988)

Reminiscences on Albert Szent-Györgyi

Denis M. Robinson

High Voltage Engineering Corporation

Albert inspired a wide spectrum of biologists; less well known is his influence on electrical engineers such as myself. Every summer Albert would fascinate me with descriptions of his recent experiments on the role of electrons in biological processes.

Equally fascinated was my wife Alix by Albert's head and what it represented. As a result, there is a bronze bust of Albert in the Meigs Room of the MBL's Swope Center. He had always refused to sit for a sculptor (the modernistic head of him in the MBL library was done by the artist from photographs). About once a year from 1973 on, Alix asked Albert, "When are you going to sit for me?" He appreciated her art but said he had for years refused all such requests. However, we were having tea together at our house in 1982 and he suddenly said, "Do you still want to do my head?" She was ecstatic. "OK, Marcia, let's come on Monday."

Alix and I had everything ready in her studio: chair on a platform at the right height, lights all ready. Marcia brought Albert in and disappeared, as did I. In a few minutes Alix came quietly out of her studio, saying, "You've got to help me. He sits with his head down in his papers and books, scanning the lines with a reading lens! I can see nothing!"

I knew what to do. I began questioning him about his early experiences in the first and second world wars. Soon he began soliloquizing with all the warmth, enthusiasm, and humor so characteristic of him. At my side, Alix began slapping clay onto the armature. I felt her satisfaction. Albert came for ten more sittings, and each time there was no problem switching him on to continue. I doubt if he was aware of Alix or the bust taking shape. He was completely absorbed in his reminiscences and obviously enjoyed having a devoted listener.

The result is the bronze bust that has adorned the Meigs Room since 1983. Replicas can be found in the Szent-Györgyi home and in the Robinson home. As far as we know, he never sat for another sculptor. Alix died in 1985. The bust speaks for itself: for me it's the result of the interaction of two people I loved. I am glad we have it.

Denis M. Robinson

Denis M. Robinson received a degree in electrical engineering from the University of London (1925–29), followed by a Ph.D. A Harkness Fellowship to MIT in 1929 gave him his first taste of America and the opportunity to do research under Vannevar Bush. Returning to London 1931, he was employed in the research labs of a large cable company, worked on TV development, and then on the development of radar.

Robinson was posted to Radiation Lab MIT in July 1941 to be the British representative on centimeter radar there and made several transatlantic trips between the centers of radar research. With the Japanese surrender he returned to the UK as head of the Department of Electrical Engineering at Birmingham University. Returning to the US in May 1946 he, with Robert Van de Graaff and John Trump, both of MIT, established High Voltage Engineering Corporation to make pressurized Van de Graaff accelerators for medicine, industry, and physics research. Using high energy electrons, they were involved with their interaction with living tissue, genetic and molecular changes. After twenty-four years as President of HVEC, he withdrew from its front line and became Chairman of the Board of Trustees of the MBL. He is currently Honorary Chairman.

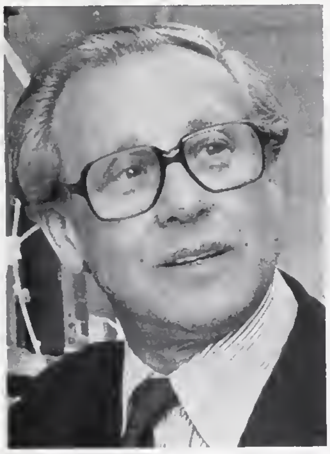
Laszlo Lorand

Northwestern University

My first memories of Prof go back to Szeged in 1943. At that time Hungary was not yet allied totally with the Axis and few had a feeling for the impending disaster. Biochemistry was taught mostly by his associates, and Szent-Györgyi gave only one or two lectures per year. His ability to communicate with students, however, was phenomenal, as many of us later witnessed in Woods Hole. I clearly recall the first lecture in Szeged in which he drew a circle, wrote down H_2, O_2, and H_2O, and then talked about oxidative metabolism. Much of it was "body language," but what made this lecture unforgettable was that upon hearing a group of soldiers marching to trumpets outside, he asked a student to close the window. He then proceeded to make some derogatory remarks about the military.

I was not introduced to Prof until late 1945 or early 1946 when Koloman Laki invited me to join the Institute of Biochemistry. My appointment was approved only after Prof saw that I was a fair volleyball player, able to approximate the skills of Andrew Szent-Györgyi on the opposite team. Through the resourcefulness of Martha Szent-Györgyi, I was allowed to take food home every evening from the Institute's kitchen to my widowed mother. This little scientific community was a veritable shelter in the storm. I pursued my medical studies and also worked in research. Prof made us take English lessons, secured opera tickets, and introduced us to prominent artists. The cultural, productive atmosphere made life bearable and hopeful again but, alas, it was not to last long.

Although Prof was responsible for bringing me to the MBL for a few summers in the early 1950's, I did not work closely with him. Nevertheless, he often shared his thoughts with me and was happy to show me new color reactions, pouring solutions from one unlabelled test tube to the other with a youthful gleam in his eyes. What a magician he was—how often I wished that he would pull yet another rabbit from the hat!



Laszlo Lorand

Laszlo Lorand was educated at the Universities of Szeged and Budapest, Hungary. While still a medical student, he joined the Institute of Biochemistry of Albert Szent-Györgyi, serving as Demonstrator from 1946 to 1948. He earned a Ph.D. in Biomolecular Structure from the University of Leeds, England, in 1951 where he worked with Szent-Györgyi's good friend, W. T. Astbury, F.R.S. In 1952, Lorand moved to the United States, and in 1955 joined Northwestern University where he now holds the rank of Professor of Biochemistry, Molecular Biology and Cell Biology.

Lorand's research interests lie at the interface between the basic sciences and medicine. He investigated molecular aspects of the complex physiological process of the coagulation of fibrinogen in blood and studied the function of the fibrin cross-linking system which is responsible for stabilizing normal blood clots and its clinical applications.

He has been a regular in Woods Hole since 1953 and is currently serving a second four-year term as Trustee at the MBL.

During the summer he deals with comparative and evolutionary aspects of blood coagulation, embryonic development, and cell-cell interactions in sponge.

Lorand received a Beit Memorial Fellowship, a Lalor Faculty Award at the MBL, a James F. Mitchell Foundation International Award, a Distinguished Career Award for Contributions to Hemostasis (Stockholm), and a Lady Davis Fellowship (Hebrew University, Jerusalem). Lorand is a member of the National Academy of Sciences.

Annemarie Weber

University of Pennsylvania

I first saw Albert Szent-Györgyi through my father's eyes. This was during World War II, probably when Szent-Györgyi began his myosin and muscle contraction work. He invited my father (Hans H. Weber) to come to Szeged for a visit. That in itself was a special event since at that time—for obvious reasons—scientific trips abroad were uncommon. My father had never met Szent-Györgyi.

My father returned with glowing reports. I was not sufficiently advanced in science then (I was either a beginning medical student or still in my last year of high school) to remember what my father said about the work in Szeged. I do remember, however, that he considered Albert Szent-Györgyi to be one of the most intuitive and imaginative scientists he had ever met.

Values then were different than today. My father was glowing with excitement because Szent-Györgyi was anti-Nazi and very open about it. Judging from the subsequent interrogation of my father, Szent-Györgyi-watching seemed to occupy much of the German officialdom's time in Hungary during those years.

Through my father's eyes I saw Albert Szent-Györgyi as an open, outgoing, and warm man—a man of extraordinary vitality and courage. He was flamboyant and incredibly charming—a kind of scientist my father had not encountered before.

Annemarie Weber

Annemarie Weber graduated in medicine and did a thesis research project with her father (H. H. Weber) on Albert Szent-Györgyi's glycerinated muscle preparation, studying the behavior of single fibers. After post-doctoral training with A. V. Hill (London) and later with David Nachmansohn (Columbia), she started studying the role of calcium and sarcoplasmic reticulum in excitation–contraction coupling at the Institute for Muscle Research in New York under Alexander Sandow. She then became a professor in the biochemistry department of St. Louis University and later, the University of Pennsylvania, concentrating on the mechanism of the calcium switch and the cooperative behavior of regulated actin filaments. She is now working on the interaction of actin and actin-binding proteins in non-muscle cells. Weber was a [illegible] of the MBL, president of the Society of General Physiologists, and a member of the American Academy of Arts and Sciences and the Academy of Science Leopoldina.

George Wald

Professor Emeritus of Biology, Harvard University

Academic scientists come every kind of way. That is to be expected: they are, as is sometimes noted, human.

But Albert Szent-Györgyi was altogether extraordinary. He was a great scientist, a great showman, an eloquent teacher and orator, a great wit, very deeply involved politically, ebullient, athletic—one exhausts the adjectives. He had life enough for three. Anecdotes, mythical and real, clustered about him. He enjoyed that, and happily contributed to them. He was the despair of numbers of "sound" men who resented his ebullience and flights of imagination, who sometimes gave Albert a bad press. Once I sat at dinner beside a very famous muscle physiologist who, when Albert's name came up somehow, turned a little red and said vehemently, "He's a charlatan!" I promptly objected; I was dying to say (but didn't) "My heavens! He's just brought your own field back to life, and you with it!" For Albert was, first and last, a great scientist. He earned his Nobel Prize three times over—of which more below.

I encountered him first in the Spring of 1933, when he lectured at the University of Zürich where I went as a post-doctoral fellow to convince Paul Karrer that it was indeed vitamin A that I had just discovered in the retina in Otto Warburg's laboratory in Berlin. Szent-Györgyi came to talk about his isolation of vitamin C. He had been chasing some strongly reducing agent in animal and plant tissues. He told us that one night they had a dinner guest who was so frightfully tedious that when he left, Albert, unready to sleep, went back to his laboratory, snatching up from the kitchen on the way a bag of paprika, which is eaten as a vegetable in Hungary. That night he found that paprika contained the mysterious reductant in especially high concentration.

Eventually he and his co-workers isolated and crystallized the substance. It was sugar-like, yet it was no known sugar. Albert, impatient, said that at first he called it *ignose;* then, a little exasperated with an editor, *godnose.* He finally accepted the editor's suggestion and called it hexuronic acid. He had found it earlier in adrenal glands, and told us that his preparation was fed to several Addison's disease patients.

I really got to know Albert when he came to lecture at Harvard a few years later. He had, with characteristic idealism, some time before prepared a whole kilogram of crystalline vitamin C that he deposited with the Health Organization of the League of Nations, requesting that samples be available to anyone ready to work with it chemically, nutritionally, or clinically. He gave me a sealed vial of his crystals, about 5 grams of them, that I still have and treasure. The crystals were light brown, not the big, beautiful, colorless crystals that soon after began to be manufactured synthetically in bulk and at low cost; but even their brownness is precious to me, like an artist's signature. Vitamin C was Albert's first great strike.

I said earlier that he earned his Nobel Prize three times over. Indeed two of those three times were cited in his Nobel award in 1937: "for his discoveries in connection with the biological combustion processes, with special reference to vitamin C and the catalysis of fumaric acid." His work with fumaric acid was an outstanding example of his creative genius.

We had entered a period in which many biochemists were busy discovering new enzymes capable of plucking pairs of hydrogen atoms out of organic molecules (dehydrogenases), the hydrogen eventually to be burned with molecular oxygen to water; and a much smaller number led by Otto Warburg with his Respiratory Enzyme (*Atmungsferment*), that he insisted alone did the burning. The Swedish biochemist Thunberg had developed a very simple procedure—we had students do it in the Physiology Course at the MBL—by which anyone could readily find scores of new dehydrogenases. Warburg, with characteristic acidity, remarked that cells were no longer big enough to contain all the known dehydrogenases. It seems strange now to realize how bitter a controversy had developed around "oxygen-activation" (the *Atmungsferment*) *versus* "hydrogen activation" (the dehydrogenases), with Keilin's newly discovered three cytochromes thrown between, hemin-proteins like the *Atmungsferment,* yet neither oxidized directly by oxygen, nor reduced directly by the dehydrogenase substrates.

Enter Albert Szent-Györgyi with a brilliant thought. In 1935 an issue of *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* appeared (v. **236,** pt. 1), its pages filled entirely with the work of Albert's laboratory all gathered under the title, "On the Significance of Fumaric Acid for Animal Tissue Respiration." There was the *Atmungsferment,* whose iron was oxidized by oxygen, and there were the three cytochromes passing that oxidation along in turn. But what was one to do with that vast array of dehydrogenases?

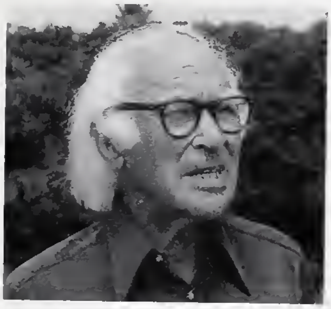
Szent-Györgyi said, let us consider only those dehydrogenases active enough to keep up with the rate of cellular respiration. At one stroke that cut the multitude of dehydrogenases down to just eight: those for succinic acid, lactic acid, glyceraldehyde (triose) phosphate, glycerol phosphate, hexose mono- and diphosphates, glutaminic acid, and citric acid. Any biochemist by now recognizes the metabolic importance of each of these substances, but in this volume they were singled out for the first time.

Of these eight dehydrogenases, it turned out that succinic acid gave up two hydrogen atoms most directly to oxygen through the cytochrome-*Atmungsferment* system, thereby yielding fumaric acid, and in turn, oxalacetic acid—a 4-carbon acid cycle acting catalytically in that only tiny amounts were needed to support respiration, these acids merely passing hydrogen without themselves being used. Together with putting the finger on citric and glutaminic acid, this series of papers marked the birth of what became the Krebs citric cycle.

But what, we wondered, was Szent-Györgyi doing during World War II? When the war ended we learned the astonishing story of the revolution in muscle chemistry that occurred in his laboratory in Szeged. After further adventures, Albert came to the United States and founded his Institute of Muscle Research at the Marine Biological Laboratory in Woods Hole. He worked hard to bring over all of his group who wanted to join him here, and he kept making astonishing discoveries—like the glycerol-extracted muscle fibers that, in the presence of the right inorganic ions would contract on adding ATP. His work here soon inspired a generation of new workers and led to developments that went far beyond muscle. That was Albert's third great strike. And with his inimitable lectures at Woods Hole and all over the world, we all enjoyed these new triumphs with him.

Albert had a wonderful gift of saying things unforgettably, of entertaining as he taught. In his Harvard lectures back in 1935 he was making the point that certain vitamins have enormous effects when fed in very small amounts, by acting as the prosthetic groups of important enzymes. He nailed the point by saying: "If a gnat could change the course of the British Grand Fleet, it could only be by biting the admiral." That kind of remark never sounded as good as when delivered with his wonderfully expressive face and gestures, and with his inimitable Hungarian accent. "Life is just the play of water," said he—the water that is split by sunlight in photosynthesis and reconstituted in cellular respiration. And then, in an ironic comment on the race for research funds under the guise of doing cancer research he remarked, "There are now more persons living on cancer than dying of cancer." "What is research?" he once asked, and answered, "It is to see what everyone else has seen, and to think what nobody else has thought."

For a long time, we lived just down the road from him summers on Penzance Point in Woods Hole. Regularly toward sunset we would see him trudging toward the end of the Point, carrying his fishing equipment; and then a little later coming back with as many striped bass as were needed for dinner. "I like to fish with a big hook," he used to say of both his fishing and his work in science. Albert was a man who liked to fish with a big hook, and he caught some big fish.



George Wald

George Wald was born in New York City on November 18, 1906. He received his B.S. from Washington Square College of New York University (1927) and his PhD from Columbia University (1932). He was a student and research assistant of Professor Selig Hecht. Between 1932 and 1934 Wald worked in Otto Warburg's Berlin-Dahlem laboratory, and there identified vitamin A in the retina. Vitamin A had just been isolated in the laboratory of Paul Karrer in Zurich, where Wald completed the identification. In Otto Meyerhof's laboratory at the Kaiser Wilhelm Institute in Heidelberg, he discovered retinal.

Wald has held appointments at Harvard University since 1934. He became an Associate Professor in 1944, was promoted to Professor in 1948, and received the Higgins Chair in Biology in 1968. He is now Higgins Professor of Biology Emeritus. Wald was the 1967 recipient of the Nobel Prize in Physiology or Medicine for his contributions to understanding the biochemical processes associated with vision and the function of vitamin A. He also received the Lasker Award among numerous other honors and awards. He is a member of the National Academy of Science and the American Academy of Arts and Sciences. Since his widely publicized talk at MIT on March 4, 1969, "A Generation in Search of a Future," Wald has spent much time and effort on what he calls "survival politics."

Ferenc Guba

Albert Szent-Györgyi Medical University,
Szeged, Hungary

I joined Prof's lab in 1941 as a student of physical chemistry, after first proving my skills in volleyball to him. Prof's charisma captured my soul, and I had admired him for his involvement in the student movement. As Rector of the University, he had established an independent, democratic, antifascist Youth Organization. As I reflect on his sparkling and charming personality, I shall relate a few personal experiences.

Soon after I joined Prof's team I had to ultrafilter protein solutions through a clay filter. On one occasion, after cleaning and burning-out the gadget, I succeeded in cracking it. I was prepared to be fired, but Prof, though somewhat unhappy, simply muttered, "One who doesn't work makes no mistakes." The atmosphere Prof created in the lab was so exciting that my job of carrying-out 200–300 monotonous viscometric measurements a day made me feel as if the elucidation of the mechanism of muscle contraction depended on my measurements.

Thinking in broader approaches to understand mechanisms of muscle contraction, Prof arranged for the late Prof. Gombás, a theoretical physicist, [illegible] us weekly seminars in quantum chemistry. [illegible] the seminars, held in a small library in a very friendly atmosphere, tea and pogácsa (an unsweetened [illegible] cake) were served at a time when tea and cake were scarce in Hungary. After the fourth seminar, the rolling blackboard was filled with differential and integral equations, and an expression of obtuseness settled on our faces. At the end of this seminar Dr. Szent-Györgyi broke the silence saying, "I have to thank the speaker for his impressive talk, but I have to confess that the only thing I understood were those three pogácsa that I consumed in the mean time." Teachers and students alike burst into laughter.

Albi hated hypocrisy; he never pretended to know what he didn't. For example when he was asked about blood coagulation, he'd reply, "Go and ask Laki."

We experienced many tragicomical situations in the laboratory. For example, the 1-liter glass centrifuge tubes used to prepare myosin eventually broke. To prevent this breakage, Prof arranged for special large condoms to be manufactured, which he slipped on the tubes. That year we each received a condom-covered centrifuge tube as a Christmas present. So we lived in Elysium.

Ferenc Guba

Ferenc Guba was born in Györ, Hungary, on March 22, 1919. He received his B.A. and M.A. in physics and chemistry from the University of Szeged, Hungary, and his Ph.D. in biochemistry from the University of Budapest. He was Assistant and Associate Professor in the Department of Biochemistry at the University of Budapest, and is currently Professor and Chairman of the Department of Biochemistry at the Albert Szent-Györgyi Medical University in Szeged. His research interests focus on the contractile proteins of skeletal muscle and the regulation of ion pumps through protein and lipid environments. Throughout his career he has received many honors and awards, including the Krusper Award and Medal for scientific achievements concerning biological structure in 1960, the Gold Medal for enhancement of sciences in Hungary in 1972, and the Jancsó Award and Medal for scientific achievements in muscle research in 1980.

Severo Ochoa

Center of Molecular Biology, Madrid

I saw Albert Szent-Györgyi for the first time in the summer of 1929. I had spent that winter in Berlin-Dahlem as a post-doctoral student in Otto Meyerhof's laboratory and was on my way to Boston to my first international congress of physiology. There were no separate congresses of biochemistry at that time. Biochemistry, at least officially, was a branch or a step-daughter of physiology.

What a thrill and what a splendid experience for a young apprentice scientist to be on the same ship with so many of the great European physiologists and to be travelling to the New World for the first time! The thrill was even greater because the young scientist was a native of Spain, a country where science had scintillated brightly only once, thanks to the genius of Santiago Ramón y Cajal, the great neurohistologist, and this had occurred long ago. The youngster had not seen great scientists before, not even the old man.

Yes, there I was sharing the Minnekhada, an old, rickety U. S. war transport ship, with some of the greatest physiologists of the world: A. V. Hill, Barcroft, Haldane, Sherrington, Szent-Györgyi, and many others. One of the stars was the bulky Jakob Parnas, the great Polish biochemist. He was very conspicuous not only because of his bulky yet handsome, attractive appearance, but because he was almost constantly jogging or otherwise exercising on deck. He was known on the Minnekhada as "*Montparnasse.*"

Szent-Györgyi was one of the most conspicuous scientists aboard the ship—so young and handsome. Where the other scientists engaged mostly in scholarly conversation, Albert was also often seen wherever people gathered to have a good time. If there happened to be music and dance, Albert would be there, dancing with the Minnekhada beauties.

Among the people travelling to Boston was a recently married Spanish couple. He was a Professor of Medicine in a provincial University; she was a real Spanish beauty. Albert used to dance with the lady quite frequently which, judging from his looks, did not make her husband very happy.

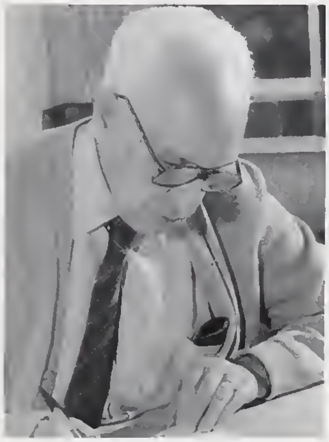
After 1929 I did not see Albert Szent-Györgyi for many years. I probably first met him in Woods Hole in 1947. From that time he always distinguished and honored Carmen, my wife, and myself with his friendship.

One could dwell on many of Albert's outstanding qualities. I remember him most for his great sense of and devotion to friendship. I know few people who can live up to the deeply human, noble virtue of understanding and valuing friendship as he did. Albert was unselfish and extremely generous. He was a real gentleman. He was charming and interesting whether he talked of science, art, music, or the beauty of nature. Carmen loved watching the lovely sunsets over Buzzards Bay with him.

Albert's lectures were always challenging and spicy. In one of the many that come to my mind he described the isolation of an anti-cancer compound from human urine. Since he used the urine of G.I.'s from a Cape Cod army camp he humorously boasted of having the American Army "pissing" for him.

Carmen and I often spent a few weeks each summer in Woods Hole, invited by the Szent-Györgyis to stay at their Penzance Point cottage or, if this was occupied by friends or relatives, at the main house. He and Martha, his beautiful, charming Hungarian wife were wonderful hosts. I recall with nostalgia the many wonderful evenings spent together either at his home, at lovely Cape Cod restaurants, or at the theater.

I will always keep a warm, happy memory of my friendship with Albert.



Severo Ochoa

Severo Ochoa, born in Luarca (Asturias, Spain) in September 1905, studied Medicine at the University of Madrid, obtaining the M.D. degree "cum laude" in 1929. The indirect influence of Cajal, whom however, he never met, led to his irrevocable decision to devote his life to the study of biology. Postgraduate studies took Ochoa successively to Berlin and Heidelberg where he worked under the direction of Nobel laureate Otto Meyerhof, and to London, where the biochemist Harold W. Dudley initiated him to study enzymology.

Back in Madrid, Ochoa was appointed Assistant Professor of Physiology in the Department of Juan Negrin (who eventually became Prime Minister of the Spanish Republic) and later promoted to Director of the Department of Physiology of the Institute of Medical Research, created by the Professor Carlos Jimenez Diaz in the new School of Medicine. In 1936 Ochoa briefly returned to Heidelberg and from there went to England to work, first at the Marine Biological Institute in Plymouth and then to the Department of Biochemistry at Oxford with Professor Rudolph A. Peters. In 1941 Ochoa moved to the United States where, after working for a year with Carl F. and Gerty T. Cori (Nobel laureates in 1947) at Washington University, St. Louis, he went to the School of Medicine of New York University. There he was (1942–1974), Research Associate of Medicine, Assistant Professor of Biochemistry, and Professor and Chairman of Pharmacology. In 1954 he was appointed Professor and Chairman of Biochemistry. In 1974 he became a Distinguished Member of the Roche Institute of Molecular Biology in Nutley, New Jersey, where he worked until December of 1985. Since then he has worked in Madrid, coordinating the work of a group of young researchers at the Center of Molecular Biology, a joint enterprise of the Consejo Superior de Investigaciones Cientificas and the Universidad Autonoma of Madrid.

Severo Ochoa has received several distinctions, among them the Nobel Prize in Physiology and Medicine (1959) and the Santiago Ramón y Cajal Prize (1982). He is a member of the National Academy of Science, the American Philosophical Society The Royal Society of London, the Leopoldine Academy of Halle (Germany), Real Academia de Ciencias (Spain), and the USSR Academy of Sciences, among others.

Ochoa has published numerous articles on the metabolism of carbohydrates and lipids and on the synthesis of nucleic acids and proteins. His investigations initiated the work that led to the unraveling of the genetic code.

Andrew Szent-Györgyi

Brandeis University

I joined the Prof's laboratory in 1945 when he moved from Szeged to Budapest. I was still a medical student, and the city had been devastated by the siege. It took some six months to rebuild and renovate the building, and install the equipment for the work to start. During this time, among other activities, Prof was reorganizing the Hungarian Academy of Sciences which initially meant providing outstanding members with food. He accomplished this by using his contacts with the Russians and money provided by an industrialist friend, Rath. Problems were occasionally unexpected. The food requested by Bekesy, who some 20 years later was awarded the Nobel prize for his work on hearing, was enormous. It turned out that he had requested food for the whole street: he was unhappy eating while his neighbors could not. Later the famous composer Kodaly asked Prof to help him retrieve the piano that Russian soldiers had taken from his apartment. Prof immediately went to the local military station demanding the return of the piano. This was not really the thing to do at the time. He asked me to accompany him and I recall vividly how uneasy I felt since the Russians were still collecting young people from the streets to fill up the POW quota they had announced prematurely. Our welcome was not very warm and the piano was returned only by contacting higher military command.

At the time, money was useless; food was the most important barter. Inflation was so rampant that I would arrange with Eva, whom I courted at the time, to meet me right after receiving my salary at the nearest confectionary where we consumed my weekly earnings on ice cream.

During those two years or so, we were fed in lieu of real pay, including provisions for one's family. Frequently, the main entree consisted of the remainders of the rabbit killed on Mondays for myosin preparation. The kitchen was supervised by Prof's wife Martha. The social center of the laboratory was the dining room where we all ate together around a large table and discussed science, politics, or whatever came to mind. Although we were of varied political persuasions, we could trust that whatever was said would stay within the room (which was important so as to avoid harassment or jail). Prof always believed in international collaboration as exemplified by science, and hated the military. He led the fight to convert the lavish Military Academy adjoining the Medical School and the clinics into student dormitories. As a non-elected honorary member of the Parliament, he proposed abolishing the Hungarian Armed Forces and recommended that the country should come under the protection of the United Nations. His comments and characterization of the behavior of the Armed Forces under German domination aroused the Secretary of Defense so much that he challenged him for a duel. This was discussed with some amusement at the dinner table. Although Prof had remarkable physical courage, he did not satisfy the wishes of the Secretary.

The Biochemistry Department at Budapest consisted of his old associate Koloman Laki who worked on blood clotting with a few associates, Ilona Banga, Ferenc Guba, Tamas Erdos from the Szeged Laboratory, and five young members working with Prof. He did not believe in "big" science, in fact, he continued working with his own hands until his 92nd year. He told a characteristic story from his Moscow visit celebrating the Anniversary of the Russian Academy in 1946, meeting his old friend Parnas, a well-known biochemist. Parnas was Jewish. The Russians saved him from the Nazi Army when the Germans occupied Warsaw using special commando groups. They provided him with a laboratory. Prof asked, "How are you set up, Parnas?"

"I have 100 people in my lab," responded Parnas.

"What are you doing?"

"Nothing."

"How come?"

"If I need anything, even test tubes," responded Parnas, "I have to fill out so many forms that it is much easier to do nothing."

We had frequent musicals on Friday evenings, performed by the best Hungarian musicians and attended by laboratory members and their guests. That is where Prof first met Eva and he liked her to the very end. Life was also colored by periodic parties and dancing. We played table tennis in one of the large corridors and there was a "compulsory" after-lunch volleyball game played in the courtyard in the full view of anyone strolling by. The political situation was getting worse and when he decided not to return to Hungary from the International Cytology Congress in Stockholm in 1947, he arranged for some of us to be invited to spend time abroad while our passage to the States could be arranged. Lorand went to Astbury, Mihalyi to Theorell, Gergely to Evans, Varga to Joliot-Curie, Rozsa to Switzerland, Lajtha to Naples, and Eva and I to Buchthal. He knew that we were in love so he wired from the US in October 1947, "Csuli and Eva should get married" which we did and never regretted. After several very happy months in Copenhagen, we arrived to Woods Hole in late June, 1948, and our American life began. Eva worked closely with Prof, assisting him with glycerol extracted psoas muscle, her greatest scientific excitement in life. We stayed in Woods Hole for 14 years making many friends and enjoying life.

Probably the greatest impact on us, taught by example, was Prof's attitude towards science. He believed that science was fun, a great adventure worth any sacrifice. Since he had a difficult time making ends meet when young, he was convinced that too much security was not neces-

Andrew and Eva Szent-Györgyi

Both **Eva and Andrew Szent-Györgyi** studied medicine at the University of Budapest. They spent 14 years in Woods Hole (1948–1962) as members of the Institute for Muscle Research at the Marine Biological Laboratory. They then moved to Dartmouth Medical School where Andrew joined Shinya Inoué's Department of Cytology as Professor of Biophysics. Since 1966 they have worked at Brandeis University on the mechanism of regulation in muscle, spending their summers at the MBL. Both are members of the Corporation. Andrew was an instructor for the Physiology Course (1953–58), was in charge of the course (1967–71), and has served as a Trustee and a member of the Executive Committee. He is a Fellow of the American Academy of Sciences, was an Overseas Fellow of Churchill College, and a Guggenheim Fellow. He has served as President of the Society of Experimental Biology and of the Biophysical Society. He has received the Humboldt Award for Senior U. S. Scientist and a Merit Award from NIH.

Editorial Note: At the time of editing this publication Eva Szent-Györgyi passed away at age 62 on March 22, 1988. Below is a photograph of her measuring tension changes in a glycerinated psoas muscle bundle soon after her arrival with her husband Andrew (Csuli) in Prof's Institute. She published under her maiden name Szentkiralyi. Eva displayed remarkable courage during her prolonged illness.

Eva Szent-Györgyi

sarily conducive for good science. The reward of science was not money, but the satisfaction of finding new relationships, discovering new facts about "life," having "big" thoughts, and making small unexpected observations using one's fingers, eyes, and even one's brain. He was willing to mortgage his home at an age over 60 when in funding difficulties to assure an additional six months of operation. He had refused to patent Vitamin C since he felt it belonged to "humanity," even though at the time he was not supported by taxpayer's monies. He was able to say at an age approaching 90: "Csuli, it could be that all I am doing is nonsense, but I have so much fun doing it."

Ferenc Brunó Straub[1]

Hungarian Academy of Science Budapest

My good fortune brought me in contact with Albert Szent-Györgyi at the beginning of 1932 and my life became closely associated with his laboratory for 15 years. There are only a few who can today recall the events of 55 years ago. I was 18 at the time, having started medical studies at Szeged University, with the intention of becoming a practitioner, to do honest work as was tradition in our family. I did not know much about the world or about science, even less about chemistry. This was all mostly obscured by a strict education at the high school level. I was encouraged to believe not to doubt things.

Then all of a sudden a whirlwind swept me off my feet: the personality of Albert Szent-Györgyi convinced me to understand and adopt different values towards the world, towards science, towards research. I believe most of the younger generation joining his laboratory experienced the same, which I am trying to describe.

The first impact was the way he gave his lectures. At the lectures I heard in Physics, Anatomy, Histology superior but mostly dull people were giving us *data* to learn, told us laws we had to memorize. Then came chemistry class where Albert Szent-Györgyi described, in simple words, the *problems,* the presently known principles, the beauty in stoichiometric reactions, and told us why the concept of pH is helping us understand the phenomena of life. Everything became clear and understandable, every new idea seemed to be further developed. We soon understood that it is not the detail, but the basic idea which had to be grasped. We realized the deep effort and enthusiasm which the search for knowledge and understanding was radiating from our teacher. During the early thirties, there were no textbooks from which one could learn a subject so as to pass an examination. The first examination turned out to be a dialogue on what we heard from Albert during the semester.

It was a bit of a surprise and a shock when Szent-Györgyi asked me whether I would like to work in his laboratory. The surprise was understandable, the shock was due to the fact I never thought of changing my chosen course, never thought of doing chemistry. However, after a few days I was convinced that working in the laboratory led by such a great scientist would be very beneficial. After about two years, Szent-Györgyi suggested that I leave my medical studies and learn some chemistry instead if I wanted to stay in biochemical research. I did not hesitate and did so.

What did I see, working in his laboratory? First of all an intensity of life and work. Prof never passed by a chance to discuss experiments, new ideas, music, politics, art, and sports. He had the natural gift of being able to talk and discuss problems with a young inexperienced man like myself, as if we were on an equal footing.

Moreover, I already realized that he liked to be contradicted in a discussion. It was, I learned, his greatest asset. When working intensively on a problem he always wanted to hear what one could say *against* the idea. This fantastically enjoyable spiritual atmosphere seemed to me worth any sacrifice. The whole atmosphere emanated from him, and I took his advice.

From an apprentice I became a scientific associate and produced papers in which I was able to contribute to an idea that Albert suggested. He was generous in giving credit to his associates. The laboratory worked mostly on the basis of a team-work. At the end of the year Albert asked everybody to write up what they did. Then he took these papers and rewrote them in a more understandable way. He wrote a summary of all these papers and described what he observed and worked out with his own hands. Then under the name of one or two of his coworkers he added the details; his name did not appear on it. It was a happy family.

A last remark of mine refers to a characteristic of Prof, evident from what I wrote about the publications. It was obvious to anybody who knew him that there was a very strong drive in him: *to understand more* about the essence of life. He often came into the laboratory excited with a new idea, saying: *now I think* I understand life. He easily attracted students and he always supplied them with promising, different ideas. He never wanted to exploit any of his students; there was no selfishness in his doings. He helped many of us to obtain fellowships abroad, and—after a few years—he did not mind if his students tried to strike out on their own, pursuing different lines of research.

Thus Drs. Banga, Laki, Vargha, Szalay, and myself later worked in different fields, with different styles and outlook. But basically what we achieved came about based on what we learned from the late Albert Szent-Györgyi, the Prof.

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[1] Reprinted with modifications from *Acta Biochim. Biophys. Hung.* 22(2-3): 135–139 (1987).

Thus in the late thirties several fairly independent lines of research were pursued by some of us. Then Prof became enthusiastic about a new idea. He saw the paper by Engelhardt and Ljubimova in *Nature* and realized the importance of the interaction between myosin and ATP. After a few preliminary observations leading to the discovery of myosin A and myosin B he suggested joining forces again on a central problem. For those of us who participated in the "Studies from the Institute of Medical Chemistry, University of Szeged" during the threatening ongoing of World War II, it was a glorious time for enjoying the spirit of discovery. Prof, the Nobel prize laureate who led this newly recreated group, was the same charismatic leader during these wartime years as he was when I first met him. Plenty of new ideas were discussed, and his attitude remained the same as before: questions have to be asked from nature, new ideas should be discussed. We felt the joy of intellectual achievement and this has given us enough moral strength, so that (with luck) we survived the final year of the war that swept our country and scattered colleagues. When peace came we had found new posts, but all of us tried to carry on the spirit of our teacher.

Ferenc Brunó Straub

Ferenc Brunó Straub was born January 5, 1914, in Nagyvárad, Hungary. He obtained a PhD in Biochemistry in 1937 from Szeged University and joined the laboratory of A. Szent-Györgyi in 1932.

Straub was an Assistant Professor (1937–1945) at the Medical Chemistry Department, Szeged University, and a Full Professor in the same department from 1945 to 1949. Between 1949 and 1970 he was head of the Medical Chemistry Department at Budapest University. He was the Director of the Institute of Biochemistry (later renamed the Institute of Enzymology) in the Hungarian Academy of Sciences from 1970 to 1985. While this Institute became part of the Szeged Biological Research Center, he was General Director of the Center between 1970 and 1978.

His studies in enzymology led to the isolation of diaphorase from muscle, lactate dehydrogenase, and malate dehydrogenase and aspartate transaminase. In the forties he isolated and characterized actin from muscle. In the fifties and sixties he studied the role of ATP in K-accumulation by erythrocytes, the SH-SS rearranging enzyme, and established the role of ascorbic acid in this process. He later was interested in the physiological significance of the fluctuation of the polypeptide chain.

Straub was Vice President of the International Union of Biochemistry from 1973 to 1976. Since 1985 he has been Vice President of the Hungarian Academy of Sciences.

Setsuro Ebashi

National Institute for Physiological Sciences, Okazaki, Japan

While working on my thesis titled "Electrophysiological approach to smooth muscle pharmacology," one day in September, 1950, I received a copy of a book, *Chemistry of Muscular Contraction* (1947) by Albert Szent-Györgyi, mimeographed on rough paper with hardly visible figures. Such a rag could not be found even in a trash can today, but it was a gem in rags. I read every page with an inexplicable excitement. I underlined many sentences and made notes on many pages. This is the only book I have read in that manner.

The second edition of *Chemistry of Muscular Contraction* (1951) arrived in Japan very soon after its publication, perhaps within a few months, indicating the improvement of the general condition of Japan in just one year. This edition described the glycerol extracted muscle preparation. These books influenced me to change from electrophysiology to muscle biochemistry. As a pharmacologist I felt comfortable studying glycerol extracted muscle bundles and later gained confidence to investigate the biochemical interactions of actomyosin.

My first personal encounter with Prof took place in July, 1959, in Woods Hole. After I discovered the ATP-dependent Ca uptake of the relaxing factor, *viz.*, fragmented sarcoplasmic reticulum, at the Rockefeller Institute under the inspiring guidance of Prof. Fritz Lipmann, I visited Prof in Woods Hole. I told him the results and discussed my ideas on Ca^{2+} in muscle contraction. He was very receptive and encouraged me heartily. I have to confess, however, that his response to the Ca^{2+} concept was rather equivocal and this disappointed me. However, he expressed his high evaluation of our work on the "relaxing factor" being a microsomal fraction. I was thrilled to meet the great man whom I had been admiring so much, and my stay of only two weeks in the Institute for Muscle Research was very fruitful.

A humble mimeocopy of Prof's book decided my destiny as it did many other Japanese scientists; it had a tremendous impact on their scientific motivations. It was truly the best seller of underground publications concerning biological sciences.

Prof visited Japan in 1969, not as a scientist but as a missionary of peace. It was just after the student's rioting and we were in despair. It was more than a comfort to see his face and hear his voice, so full of life—it was almost the salvation of our spirit. Kosack Maruyama and I had the pleasure and honor of accompanying him for several days in his trip to Hakone, a resort with a hot spring. Quite unexpectedly, he frankly told us many secrets and anecdotes on his life. Perhaps we should recall his words as far as possible and record them in memory of our Prof. Here I describe only a short sentence: "Important things cannot be done by a single substance, but by two, like myosin and actin."

Setsuro Ebashi

Setsuro Ebashi was born in Tokyo on August 31, 1922. He received his M.D. (1944) and D.Sc. (1954) from the University of Tokyo. In 1959 he was appointed Professor of Pharmacology at the University of Tokyo and remained there until 1983 when he accepted an appointment as a professor at the National Institute for Physiological Sciences. He is currently Director of this institute in Okazaki, Japan. Among his many contributions to muscle biochemistry are the discoveries of the ATP-dependent uptake of calcium by the sarcoplasmic reticulum and the troponin system in the regulation of actin-myosin interaction. Ebashi is a member of the Japan Academy, and a foreign member of the Mitglieder der Deutsche Akademie Leopoldina, the American Academy of Arts and Sciences, the Royal Society (London), the Belgium Royal Academy of Medicine, and the Academia Nationale del Lincei (Rome). He received the Imperial Prize from the Japan Academy and the Peter Harris Award. He was decorated with the Order of Cultural Merit (Bunka-Kunsho) in 1975.

Salvador E. Luria

Massachusetts Institute of Technology

Salvador E. Luria

Salvador Edward Luria was born in Torino, Italy, in 1912. He graduated *summa cum laude* from the University of Torino Medical School in 1935, and became a naturalized American citizen in 1947. He received the Lepetit Prize in 1935, the Lenghi Prize in 1965, and was awarded the Nobel Prize for Physiology or Medicine in 1969. Other highlights of Luria's career include two Guggenheim Fellowships and a Non-Resident Fellowship from the Salk Institute for Biological Studies. He was a Professor of Bacteriology at Indiana University and the University of Illinois before he began his work at the Massachusetts Institute of Technology as a Professor of Microbiology in 1959. From 1964 to 1974 he served as a Sedgwick Professor of Biology. Presently, Luria is Director of MIT's Center for Cancer Research and an Institute Professor Emeritus.

John Gergely

Boston Biomedical Research Institute

Albert Szent-Györgyi not only started me on what became a life-long occupation, or shall I say avocation—research on muscle—but he remained a constant influence throughout my scientific career. I vividly remember two aspects of Prof's work that made a profound impression on me at the time I was finishing medical school or shortly thereafter. One was a paper in *Science* in which Prof described his ideas about the application of quantum mechanics to the understanding of biological phenomena. The other was a series of publications on the pioneering work on muscle, from the Szeged Institute, that laid the foundation for much that we know today about muscle structure and function.

Beyond the immediate influence in scientific matters, I remember Prof's philosophy about the unity of all living things, his equating of truth and beauty in science. I remember his telling me at one point that while living and working in Woods Hole he couldn't help noticing the soaring seagulls and seeing perfection and beauty in these creatures. He said that in the face of such beauty he would be ashamed of doing poor research—not that he ever could.

John Gergely

John Gergely graduated in medicine from the University of Budapest in 1942, and became a member of Szent-Györgyi's Budapest Institute in 1946. At Szent-Györgyi's suggestion, he studied quantum chemistry with the late M. G. Evans in Leeds as a post-doctoral fellow. Szent-Györgyi helped him move to the National Institutes of Health in Bethesda, Maryland, where he began his research on muscle proteins. Since 1951 Gergely has had appointments at the Massachusetts General Hospital and on the faculty of Harvard Medical School. In 1961 he joined the Retina Foundation (now known as the Boston Biomedical Research Institute) where he organized the Department of Muscle Research and is working on the structure and function of the proteins of the contractile and regulatory machinery of muscle.

Teru Hayashi

Professor Emeritus, University of Miami School of Medicine

I first met Prof in New York in 1947 or perhaps 1948. He came to Columbia seeking help to arrange for a number of his colleagues and students to get out of Hungary and Europe. I was asked to assist Prof, and did my best to arrange teaching positions for his students so that they could obtain the necessary visas. Eventually they wound up teaching at the New School in New York, and I buried myself in my duties at Columbia and forgot about the visiting Hungarians. But Prof never forgot, and much to my surprise invited me to Woods Hole the following summer to a cottage he had rented for my family and me in West Falmouth. I suppose he felt that he owed me this (he did not) and this was his way of extending a hand of friendship.

That summer I began to learn that Prof was not the cooly intellectual and analytical type that scientific geniuses are supposed to be, but rather a warm, compassionate individual who loved people—a real Mensch. Oh, he was deeply involved in science, but he never lost sight of people. That summer he would invite a number of people in for informal seminars on Sundays at his newly acquired home on Penzance Point. We would begin about 9:00 am and go on 'til noon, when there would be a long break for lunch and a swim on the beach until about 3:00 pm. We would then resume until about 5:30. It was a very civilized way to run a seminar, I thought. There was one other feature that I thought was unusual. At the meetings, there were several very attractive young ladies who made for good company at lunch and on the beach. I asked one of them what she majored in in college, and her answer was "Fine Arts." When I asked her how she came to be in a biochemistry seminar, she simply said that she (and the others) had been invited; the scenery at the seminar had been considerably enhanced.

One summer Prof learned that Woods Hole was a great place for game-fishing, especially for striped bass, and suggested that I teach him the fine points of striper angling. So one beautiful August morning at 5:30 am we were in a boat out in the Hole greeting the sunrise with me showing Prof the flow of the tide and the location of the rocks and the best places to cast and to troll. Unfortunately, there were no fish biting that morning, but we had three hours of rich conversation together.

A few weeks after that fishless morning, Prof went casting from Penzance Point and caught a striper that was twice as big as anything I had ever hooked (40 pounds). "Just lucky," I thought enviously. But later, in a lecture to visiting students at the MBL, he told this story. "When I go fishing here," he told them, "I put this big hook on my line." Here he drew an enormous hook on the blackboard. "All the locals would laugh at me, saying 'you'll never catch any fish with that hook.' But I didn't mind. I knew I wasn't going to catch any fish anyway. But it is much more exciting NOT to catch a BIG fish than not to catch a little fish" His message to the students—"go into everything whole hog. Shoot for the stars."

He also made a much broader impact on Woods Hole social life with his parties, which were really "whole hog" affairs not only for the numbers of people involved but for the thematic basis for the parties. There were costume balls, and one famous party for the discovery of DNA structure featuring Jim Watson both in person and in effigy. They were wonderful parties.

It was in 1964 or thereabouts that he went "whole hog" into social and political issues at local, national, and international levels. He spoke in public of his concern with nuclear armaments, environmental pollution, the building of a nuclear power plant on Cape Cod, etc. One night that summer he asked me over to read some "poems" he had written and to tell him what I thought of them, since he was thinking of submitting them for publication. They were in the form of prayers; as poetry, I felt that they were not very good, but not wanting to hurt his feelings, I diplomatically (!) asked, "Prof, do you always publish everything you write?" I have never forgotten his answer, which was really a statement of his committment to the intellectual life. He said, "Tay, I have trained all my life to think, and the only thing I produce as a result are thoughts and ideas, and these must be published if they are to survive, otherwise they die and fade away in my brain." The prayers were published in 1970 in Albert's book of essays, *The Crazy Ape*.

These are some of my treasured remembrances of Albert Szent-Györgyi, great scientist, teacher, lecturer who became these things through a whole-hearted commitment to everything he did.

Teru Hayashi

Teru Hayashi was born in Atlantic City, New Jersey, in 1914. He received his bachelor's degree in 1938 from Ursinus College in Pennsylvania, and his Ph.D. in 1944 from the University of Missouri. He taught physics in the Army Air Force, Army Special Training Program until 1945, and soon after became an Instructor of Zoology at Columbia University. He was promoted to Professor in 1958 and then to Chairman of the Zoology Department in 1965. Hayashi served as Professor and Chairman of the Illinois Institute of Technology's Biology Department from 1967 to 1979, and then worked as a senior scientist at the Papanicolao Cancer Research Institute (University of Miami) until 1985. Hayashi's research has focused on muscle proteins, particularly on the various states of actin and its interaction with myosin. Since 1983 he has also been a Professor Emeritus of Biochemistry at the University of Miami School of Medicine. Hayashi first came to the Marine Biological Laboratory in the summer of 1939, sponsored by Dr. L. V. Heilbrunn. He became a member of the Corporation circa 1945 and is presently a Trustee Emeritus.

Ronald Pethig

University of Wales, Bangor

By chance I read an article titled "The Study of Energy-Levels in Biochemistry" by A. Szent-Györgyi

(*Nature*, August 9, 1941), that described more clearly than my lecturers had done the concept of electron energy bands in crystals. Furthermore, these concepts were being applied to the understanding of biological activity some six years before their practical application in transistors. The article concludes with the statement: "Biochemistry is, at present, in a peculiar state. By means of our active substances we can produce the most astounding biological reactions, but we fail wherever a real explanation of molecular mechanisms is wanted. It looks as if some basic fact about life were still missing, without which any real understanding is impossible. It may be that the knowledge of common energy-levels will start a new period in biochemistry, taking this science into the realm of quantum mechanics."

At that moment, I knew the path I wanted to follow. Thirteen years were to pass before I had the opportunity to meet "Prof."

Prof was approaching his 82nd birthday when I could witness first hand his energetic and philosophical research style. Within minutes of first setting foot in his laboratory he had Jane McLaughlin helping in the preparation of a dazzling array of color-producing reactions. Most of all I was intrigued by his preparations of colored proteins. Back in Wales, my young colleague Peter Gascoyne was in the second year of his Ph.D research into the dielectric and hydration properties of protein charge-transfer complexes. At that time the best we could manage was to change "white" proteins into ones having a slight purple tinge. Here I was being shown yellow, brown, and even black proteins, prepared under gentle conditions. A black protein structure suggested one containing a large number of free radicals. Could the energy levels available to these unpaired electrons possibly be such as to allow them a significant degree of delocalisation? I was to return to Woods Hole five weeks later with Peter Gascoyne. As an indication of Prof's remarkable energy and the scope of his ideas at that time, I can quote directly from three of his letters written, before our arrival, all on the same day (September 8, 1975).

"Since you left I have made one rather exciting observation. I found the free radical of ascorbic acid, and think that it plays an important role in electron transfer from protein to O_2."

"I would like to know urgently whether the red juice gives an ESR signal. The white fluid is the pure solvent—for a control. If there is a result please let me know by wire (collect)."

"I have forgotten to add to my letter in the package that the solvent was dimethyl-sulfoxide, and the purple material probably the free radical of ascorbic acid."

Eight days later, on his 82nd birthday, he wrote:

"I thank you also for the ESR measurement on the two juices I have sent you. The result is most enjoyable, and also most important."

Needless to say, we had found the free radical he expected. I still remember those samples with their deep red color. Prof loved colored solutions. Later he writes:

"I am getting more and more interested in SH, which is intimately connected with the problem of protein radicals. It is too complex, and I am too much at the beginning to write about it. I will tell you about it at Sanibel. There is an old Viennese saying: 'Everything has an end, only the sausage has two.' The radical story has two ends too, a dicarbonyl and an SH end."

I would like to think that we made progress in making links between Prof's bioelectronic ideas and cell biology. At the very least we came to recognize the promising and the not so promising trails.

Until illness finally slowed him down, Prof tackled his science with vigor, integrity, and humor. When experiments came up with an unexpected or "wrong" result, to him that was "amusing." I would like Prof to have the last words (from a letter of April 26, 1985):

"When I settled here in Woods Hole and invited you later I felt that I had started something, only I did not know what. Now it is becoming more or less clear what this was: a shifting of the grounds on which biology stands to submolecular reactions. The visit of Jonathan to my lab brought in a new factor into this situation which we did not consider before: the charge of the whole cellular system. And this is terribly important. Much of my earlier work considered the redox potentials. The free energy changes of oxidoreduction, which dominated my work, is but one aspect. So this is the situation Ron. I did not know that this will develop when you disembarked in Hyannis to discuss with me the yellow casein. It is amusing and exciting."

Ronald Pethig

Ronald Pethig was born on May 10, 1942 in Ripley, Surrey, England. He obtained a B.Sc (1963) and Ph.D (1967) in Electrical Engineering from the University of Southampton, and a Ph.D (1971) in Chemistry from the University of Nottingham. He was appointed Lecturer (1971), Senior Lecturer (1978), and Reader (1982) in Electrical Materials Science at the University of Wales, Bangor, and awarded a Personal Chair in 1986. He was appointed Director of the Institute of Molecular & Biomolecular Electronics, Bangor, in 1986. In 1982 he was elected to the Corporation of the Marine Biological Laboratory in Woods Hole.

Peter Gascoyne

University of Texas System Cancer Center, M.D. Anderson Hospital

Imagine how it felt for an English country boy to find himself bound suddenly for a village in an immense foreign country soon to encounter a man reputed to be one of the giants of twentieth century science. I remember the warm grip of Prof's handshake, his excited, smiling face, and his warm hospitality. His greeting to me was typical of his totally unassuming nature, his understanding that what makes a man is not his past but what he is now. Unknown to me then, I would return the following November to spend the next ten years of my life working with him.

Everyone who visited Prof remembers his joyful demonstrations of colorful test-tube reactions beside the window overlooking the ferries. He only permitted experiments on the spectrometer after he had calibrated things by eye. His experimental ability was intuitive and uncanny, and his infectious wonderment at the living world never dimmed. More than anything, I remember the long hours we spent at his desk discussing ideas, and the "pow-wows" with visitors out at Penzance Point in the summer. Seldom did he give close discussion or criticism of experiments except to eagerly embrace any new data and incorporate it immediately into his thinking. It was the idea that meant everything to Prof, and he was always convinced that as soon as you had the correct idea the experiment would be simple. To him, fishing with a big hook meant using a big idea as bait.

It was not always easy working with Prof, for I was schooled in the science of careful stepwise experiments. He thought that approach was a waste of time and never managed to relate to the fact that some experiments take weeks on end. Prof was like an impressionist painter, free to move about the canvass at will and to paint with bright colors and a big brush. All too often those who read his papers looked skeptically at the details which were often as meaningful as the microscopic detail on a Monet; all too often they missed the wood for the trees. Perhaps their criticisms were sometimes justified, but Prof's track record ended up looking better than theirs' and he had a heck of a good time too.

I owe a lot to Prof. He taught me it is alright to let your imagination wander; it is alright to be what you want to be; it is alright to be wrong.

Peter Gascoyne

Peter Gascoyne was born in Drayton, England, on January 10, 1952. In 1970 he entered and later received his PhD from the University College of North Wales, Bangor, after doing a research project on electronic and protonic mobility effects in proteins. He moved to Woods Hole, Massachusetts, at the end of 1976 to work with Albert Szent-Györgyi at the MBL on the charge-transfer properties of proteins. Gascoyne remained in Woods Hole, investigating the bioelectrical properties of cancer cells, until Szent-Györgyi's laboratory closed in September 1986. He is currently an Assistant Biochemist and Assistant Professor of Experimental Pathology at the University of Texas System Cancer Center, M.D. Anderson Hospital, in Houston, Texas. He is now investigating electrically modulated membrane signalling processes in normal and neoplastic cells—a direct outgrowth of his studies with Szent-Györgyi and their earlier collaborations with Houston.

Zoltan Bay

The American University

Not being a biologist, but a lifelong friend of Albert, I will recall only a few episodes exemplifying his human greatness. I have been a witness of Albert's life since the early 1930's when we were both appointed professors to a newly organized university in Szeged, Hungary. He was a young man, who even looked too young for his age but who was very well advanced in his research work. He was open minded and frank in expressing his opinions and that was when the troubles first began. It was soon suggested that that childish looking young guy ought to keep his mouth shut. When he was first recommended for election to the membership of the Hungarian Academy of Sciences, he was voted down. And when he received the Nobel Prize, it was a shock to the old academicians. In fact one of them insisted that the Nobel committee was wrong for not giving him the prize instead of Albert. Of course, that good man forgot that his name never was known outside of Hungary.

Naturally, the Nobel Prize gave Albert an exceptional standing in Hungary, even in political life; and that was very much needed in the years following the outbreak of World War II. In those years Albert opposed the Nazis, both the German and Hungarian Nazi atrocities.

When the Hungarian government realized that the Germans were going to lose the war, Albert was asked to go to Istanbul to initiate contact with the British. His dealings with British authorities in Turkey were discovered and reported by German spies and on his return to Hungary, Albert was forced to go into hiding because the Gestapo was looking for him.

While in hiding from the Gestapo, he secretly joined an underground Resistance Front organized by a coalition of left wing political parties and a patriotic part of the Hungarian Army. The goal of the Front was to avoid the siege and sacrifice of Budapest by preparing a collaboration between the advancing Red Army and the anti-Nazi part of the Hungarian Army.

Albert was asked to fly over to the Russians (a very risky mission) carrying a memo from the Hungarian Front to begin arrangements between the two Armies. The Hungarian aim was to declare Budapest "an open city." A connection by radio between Albert and myself was arranged by a team of radio engineers working in a research laboratory that I headed at the time.

Two days before Albert's departure, the entire resistance movement was betrayed and reported to the Germans by traitorous Hungarian spies built into the organization. The participants of the movement were arrested. Albert was saved by hiding at the Swedish Embassy. I was arrested but freed by two members of the German Industry Commission in Hungary who at that time already wanted to acquire "good points" for an anticipated trial after the war. The main organizers of the resistance movement were executed.

Probably the only time in Szent-Györgyi's life that he was not forced to be in opposition to society was during a few years after World War II. In those few years, he attempted to form a society based on advanced democratic principles.

First, there was a short time span of a few months when the dream of many good people was that in the democratic republic of Hungary Albert would be the president, following the pattern of Poland and the famous pianist-president Paderevsky.

History ran in a different direction. Instead of a democratic republic in the western style, a "peoples' republic" was being formed, taking over many forms of political and social life from the Soviet Union.

In spite of such political changes, Szent-Györgyi threw himself with great enthusiasm into the process of building a new cultural life in the Hungarian Society. He organized a Supreme Educational Council in the country and started to reform scientific life by reorganizing the Academy of Sciences. The latter task turned out to be nearly impossible: the majority of the old-age members refused to give up their conservative views. Thus, Albert founded a new Academy of Natural Sciences. Finally, a fusion of the two Academies was worked out by the Hungarian Government which at that time was more advanced in modern democratic principles than the old fashioned Academy.

It is characteristic of Szent-Györgyi that while he was asked to become president of the reformed Academy (created in the most part by his endeavors), he resisted and recommended instead of himself Zoltán Kodály, the great composer and founder of a new school of musical education, for president. The cooperation of the two great men, Kodály and Szent-Györgyi, was of great benefit for the Hungarian culture.

But not for long, Hungary became dominated by an oppressive dictatorship organized by Stalinist Rakosi. Many good scientists of Hungary decided at that time (around 1948) that they could not continue their scientific work amidst such circumstances and escaped from the country. Albert also left and came to Woods Hole.

Zoltan L. Bay

Zoltan Lajos Bay was born in Gyulavari, Hungary, in 1900. He received his MS degree in physics and mathematics in 1923 from the University of Budapest and his Ph.D. degree in physics in 1926. He began his career in 1930 as a Professor of Theoretical Physics at the University of Szeged, Hungary, and served as a Professor of Atomic Physics at the Technical University of Budapest from 1938 to 1948. Bay was a Research Professor of Electronics at The George Washington University in Washington, DC, until 1955 when he became a Physicist for the National Bureau of Standards. Since 1972 Bay has been a Senior Research Scientist at American University in Washington, DC. Highlights of his achievements include the first experimental proof that active nitrogen contains free nitrogen atoms, and the first use of secondary electron multiplication for particle counting.

Tamas Erdos

CNRS, Gif-sur-Yvette, France

I will refer to a few events covering a fifty-year period of Szent-Györgyi's life.

In 1937, the celebration of Prof's Nobel Prize award took on the dimensions of a National Holiday in Hungary. The night of the award, the students of the Szeged University greeted him with hundreds of torches; the next morning, we covered the bench in the lecture room with flowers.

In the forties Prof was already working on muscle proteins. In 1947 Prof sent me to Svedberg's laboratory because Svedberg complained that they were unable to obtain myosin "crystals." "In our lab the youngest technician repeats this preparation every day. You will be able to do it as well," Prof said. And he sent me off. In Svedberg's laboratory we took one of the first electronmicrographs of myosin "crystals."

In Paris my wife, Agnes Ullmann, worked with Jacques Monod and we realized suddenly that Prof and Monod, these wonderful people having so much in common, had never met. We organized the meeting, and it was an immediate success—they called each other "Alby and Jacquot. . . ."

Ten years later, after the celebration of his 82nd birthday at Boston, some close friends were invited to his home, where he announced his marriage. He was a marvellous host, preparing the breakfast himself.

For Prof's 90th birthday, Andras Beck, the famous Hungarian-born Parisian sculptor, wanted to make a medal. He spent a few happy days at Prof's place where they became friends. The result was a beautiful medal. One face of the medal shows the chemical formula of vitamin C and its source: the paprika of Szeged. On the other face, the noble and impressive face of Prof—with his little mocking smile—has been immortalized by the masterly hand of the artist.

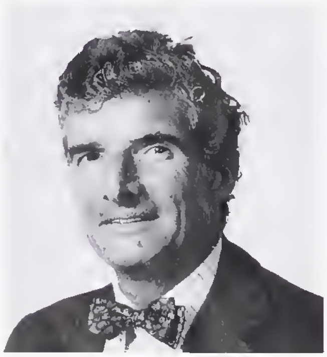
Tamas Erdos

Tamas Erdos was born in Budapest, Hungary, in 1919. He joined Szent-Györgyi's group in 1937 while studying medicine at the University of Szeged, Hungary, and received his M.D. in 1944. He studied muscle proteins until 1954 in Szeged, and later in the laboratory of Svedberg and Tiselius (Uppsala, Sweden) and in Budapest. His focus then changed to the study streptomycine's mode of action. During Erdos' association with the Laboratoire d'Enzymologie, CNRS, Gif-sur-Yvette, France (1964–1979), he studied the biology of reproduction. He continued his research on this subject in collaboration with A. Csapo at Washington University in St. Louis, Missouri, until 1986.

Prosser Gifford

Marine Biological Laboratory

Whether he was swimming through the Hole, riding his motor-bike on Penzance Point, or fishing for blue fish, Albert showed as much restless intellectual energy and physical drive in his avocations as in his science. There was always a passionate, and always an inquiring dimension to everything he did. When he learned to sail his small boat, he persisted until it was half-full of water and its lines in a hopeless tangle. He radiated enthusiasm for the central purpose of whatever he was about and had little patience for trivia. Yet he could illuminate complex ideas in simple, powerful language. As a friend, a neighbor, and a colleague he was a stimulating presence—simultaneously considerate, assertive, and unforgettable.



Prosser Gifford

Trained in literature, law, and history, **Prosser Gifford** has taught at Yale and Amherst, where he served for 13 years as Dean of Faculty. In 1980 he was appointed Deputy Director of the Wilson Center in Washington, DC. The Wilson Center sponsors and supports advanced research in the humanities and social sciences. A third-generation summer resident of Woods Hole, Mr. Gifford became Chairman of the Marine Biological Laboratory's Board of Trustees in 1978.

Irving M. Klotz

Northwestern University

For decades Prof's annual lecture in the MBL physiology course was the most popular academic event of the summer in Woods Hole, for students, investigators, local residents, and visitors. With his lively technical exposition interlaced with metaphors, such as "fishing with an enormous hook," he imparted an unabatable excitement that stimulated all within his reach. This he tempered with parabolic stories, such as that about his isolation of a new natural product as beautiful crystals, which turned out to be derived from impurities in the rubber tubing of his extraction apparatus. His was a restless, ever-curious mind. As he said in one of his essays, his brain reminded him of a popular Hungarian laxative that used the slogan, "While you sleep it does the work."

Our generation was privileged to feel his enthusiasm and to absorb his insights directly. It is saddening to realize that our descendants will be deprived of his wisdom and humor.

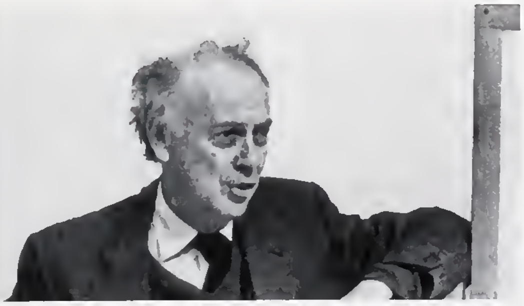
Irving M. Klotz

Irving Myron Klotz was born in Chicago, Illinois, in 1916. He received his bachelor's degree from the University of Chicago in 1937 and his Ph.D from the same institution in 1940. Soon after, Klotz began his career at Northwestern University as an Abbott Fellow and Research Associate. He then became an Instructor for the Chemistry Department in 1942. Since that time, Klotz has filled a number of faculty positions at Northwestern in the disciplines of chemistry and biology, and is currently a Professor of Chemistry and Biology, Chemistry and Biochemistry, and a Morrison Professor. He has published in scientific areas ranging from limnology to astrophysics, but his primary focus has been on the structure and function of biological macromolecules, ligand-receptor interactions, and biomolecular energetics. He also has a strong interest in the creative process in science. Klotz has received many honors and distinctions throughout his career, including two Lalor Fellowships, the Eli Lilly Award from the American Chemical Society in 1949, and the Midwest Award in Chemistry in 1970. He is a fellow of the American Academy of Arts and Sciences, the Royal Society of Medicine, and a member of the National Academy of Sciences. Klotz has long been a corporation member of Marine Biological Laboratory, serving as a Trustee from 1957 to 1965, and a member of the MBL's Executive Committee from 1961 to 1965.

James D. Watson

Cold Spring Harbor Laboratory

My book *The Double Helix* had its origins in late August 1962 in the little study off the entrance to "Seven Winds." By then Marta had accepted my lack of charm and had let me know that I was always welcome to stay with Albert and her when I needed to escape from Harvard. Earlier that year, I had started thinking that the story of how the double helix was discovered might make a perfect *New Yorker* series and I drove down to Woods Hole in my little MGTF thinking of future fame as a writer. That night Marta and Albert were hosting a benefit for the Harvard history professor Stewart Hughes, then running for senator as an independent alternate to President Kennedy's then dimly perceived younger brother Teddy. After a Landfall supper of steak and white wine, I began looking for an opening paragraph that would let me go on. I started musing about Francis Crick and soon I had written down "I have never seen Francis in a modest mood." The first chapter didn't get finished until my return to Harvard, and I remember thinking that Albert and Marta, knowing Francis well from the Gamow summer of 1954, would especially like it. Unfortunately, when I next saw Marta the following June, she was mortally ill with cancer, and I never had the chance to show her that I might have more talents in describing genius than in being confused for it.



James D. Watson

James D. Watson is best known for his discovery of the structure of DNA (deoxyribonucleic acid), for which he shared with Francis Crick and Maurice Wilkins the 1962 Nobel Prize in Physiology or Medicine.

Born in Chicago, Illinois, in 1928, Dr. Watson received a B.S. (1947) from the University of Chicago and a Ph.D. (1950) from Indiana University, both in Zoology. Following a National Research Fellowship in Copenhagen and a National Foundation of Infantile Paralysis Fellowship at the University of Cambridge, England, he spent two years at the California Institute of Technology. He joined the Harvard faculty in 1955 and became Professor in 1961, resigning in 1976 to become full-time Director of Cold Spring Harbor Laboratory.

Dr. Watson was awarded the John Collins Warren Prize of Massachusetts General Hospital (1959), the Eli Lilly Award in Biochemistry (1960), the Albert Lasker Prize, awarded by the American Public Health Association (1960), the Research Corporation Prize (1962), the John J. Carty Gold Medal of the National Academy of Sciences (1971), and the Presidential Medal of Freedom (1977).

His memberships include the American Academy of Arts and Sciences (1958), and the National Academy of Sciences (1962). He holds honorary affiliations with the Danish Academy of Arts and Sciences (1963), Clare College, Cambridge University (1968), Athenaeum, London (1980), and the Royal Society, London (1981).

Linus Pauling[2]

Linus Pauling Institute of Science and Medicine

Albert Szent-Györgyi may well have been the most charming scientist in the world. His charm was demonstrated in his personal contacts and also, together with his originality and enthusiasm, in his lectures and writings. His personality may be illustrated by a story told to me recently, forty-five years after the event, by my daughter. She met Szent-Györgyi when she was five years old, and was captivated by him. He entered into a conversation with her, treating her as an adult, and he also gave her a doll. She retains a vivid memory of the occasion. Before his arrival, my wife and I told her that Dr. Szent-Györgyi was coming to dinner with us, in Pasadena. She could not understand why a living person should be called a Saint. During his visit she decided that if anybody should be called a Saint, it is Szent-Györgyi.

However, it is not for his saintliness (about which there might be some doubt) or his charm that we honor and remember him, but rather his great contributions to science. Few investigators have made such important discoveries and have contributed so much to the development of biochemistry as Szent-Györgyi.

Linus Pauling

Linus Pauling was born in Portland, Oregon, in 1901. He received his bachelor's degree in 1922 from Oregon State College and his Ph.D. degree in chemistry in 1925 from the California Institute of Technology. He was awarded the Nobel Prize in Chemistry for 1954 and the Nobel Peace Prize for 1962 and has received many other awards in the fields of chemistry, mineralogy, biology, medicine, and peace. Presently he is Senior Research Professor at the Linus Pauling Institute of Science and Medicine, Palo Alto, California.

John T. Edsall

Professor Emeritus, Harvard University

Back in the 1920s, while still a student at Harvard Medical School, I started work in its Department of

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[2] Text reprinted with modifications from *Foundations of Physics* **13**(9): 883–886.

Physical Chemistry, under the guidance of Edwin J. Cohn, on a protein extracted from muscle, which we then—following Willy Kühne and others—called myosin. This interesting protein became extremely interesting when Alex von Muralt arrived from Switzerland, to study muscle birefringence, and proceeded to show that our "myosin" was indeed strongly birefringent in solution, when the highly elongated molecules became oriented in a velocity gradient. The two years that Alex and I devoted to the subject were among the most memorable of my life. Albert Szent-Györgyi was unknown to us at that time, and indeed for several years thereafter, but the time would come when he would make discoveries that would profoundly change the interpretation of what we had done, and reveal new aspects of fundamental importance.

That, however, came later. It was Albert's work on vitamin C that first brought him to the attention of the world. He was already famous for this when he first paid a visit to Harvard—in 1935, I think—and gave three lectures. He had an important story to tell, and it centered on his pioneering work on the role of dicarboxylic acids in intermediary metabolism—important work indeed, which was later included, along with his vitamin C work, in his Nobel Prize citation. It was an essential prelude to the tricarboxylic acid cycle of Hans Krebs. Albert was a man of infinite jest, and his lectures were constantly enlivened with humorous interludes. Indeed, I remember one distinguished member of the Biology Department remarking "It's a good substitute for Mickey Mouse." The Department was thinking of appointing a Professor of Biochemistry—they had no one so labeled at that time—and Albert was obviously being considered for the job, but evidently no offer came through. The Mickey Mouse image probably damaged him in the eyes of the senior professors. Indeed, Albert in those days had the gift, perhaps unfortunate for him, of telling about his work in terms that made it sound improbable and fantastic to many of his listeners. There was always a solid basis of new insight and important work behind it all, as the scientific world came to appreciate before long.

Anyway, back at home at the University of Szeged, he was tackling the deeper and far more difficult problem of muscular contraction. He extracted myosin with potassium chloride solutions, as I had done years before under Cohn's guidance; but he saw what I had failed to see: if the extraction was prolonged, the preparation became more viscous and sticky, though not much more protein was extracted. That was the beginning of the discovery of actin and actomyosin, which F. B. Straub, in Albert's laboratory, was to carry through to completion. It opened a new world for muscle biochemistry. All this was being done in the shadow of war, and of the Nazi domination of Central Europe. And Albert was constantly in peril of his life because of his contacts with the British. It was only at the end of the war that the muscle work, with all its far-reaching new findings, reached me. I had been far away from muscle, working on blood plasma, and especially blood clotting, in the hectic war years.

Albert's was a restless spirit. After he came to the United States and settled in Woods Hole, he soon abandoned the study of muscle and concentrated on "submolecular biology" and electronic energy levels. He never pretended, I think, to have made any deep study of quantum mechanics, but he aimed to stimulate other people to take account of new developments that he recognized as important for the future.

His ideas on cancer research never seemed really promising to me, and I was distressed when the NIH insisted on my serving on a panel that was to visit his laboratory and evaluate the quality and promise of his research. We regretfully came to the conclusion that the underlying conceptions did not look promising compared to research that was going on elsewhere. We did recommend some continuation of funding, but of course Albert wanted funding at a far larger level. I felt, with pain, that he had lost the special touch and instinct that had guided him aright in his brilliant pursuit of significant problems in the past.

However, I will not end on this note. He was a deeply human man, whose life was repeatedly torn by the terrible upheavals and inhumanities of our time. He had a vision of what humanity at its best could achieve, to which he held, although at times he certainly despaired of the follies of mankind. He was a unique and unforgettable man. The world was a better and livelier place for his contribution to it.

John T. Edsall

John T. Edsall was born in Philadelphia in 1902, but since the age of 10 has been a New Englander. He received his A.B. (1923) and M.D. (1928) from Harvard, and spent two years at University of Cambridge (1924–26) working chiefly in biochemistry. He was a tutor in Biochemical Sciences at Harvard (1928–68) and researched proteins in the Department of Physical Chemistry at Harvard Medical School (1926–54). Edsall served as an Assistant Professor of Biochemistry (1932–51), a Professor (1951–73), and is currently a Professor Emeritus at Harvard University. He is now chiefly concerned with history of modern biological sciences and social problems related to science.

Michael Kasha

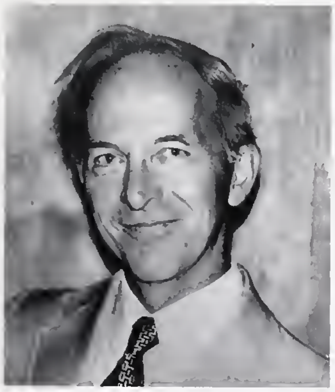
Florida State University

I had the occasion to visit Albert Szent-Györgyi in late summer just before his ninetieth birthday. I had been visiting Seven Winds, his rambling old Cape Cod house on Penzance Point in Woods Hole, many times since 1957 when I first met him. He had come to Tallahassee to learn about molecular triplet states, and I in turn had visited him to give several weeks of morning lectures to his research group. In later years in participating in his lively summer conferences, to which he invited a variety of stellar researchers, I had frequently gone to his MBL lab and watched him work at the bench. Starting with the neatest preparation set out by Jane McLaughlin, his longtime and faithful lab assistant, an awful mess developed, but inversely proportional to more and more dazzling, original experiments. I found myself always thinking; "Why hadn't I thought of doing that?!"

So this Sunday morning at the end of August before his 90th, after an initial morning coffee, Albert said, "Mike, let's go to the laboratory!" I admit at being surprised, as I had thought of making only a social call.

We quickly reached the laboratory, and sitting at the windows facing the deep blue colors of the Woods Hole gap, Albert started setting up an experiment. I thought to myself, what an inspired man. Here he is, sitting at one of the most beautiful waterfront vistas in the country, on a brilliant Sunday morning, and he is focussing on a series of test-tube experiments. He gives me a brief lecture on dihydroxybenzene derivatives found in wheat germ, and how he thinks there is a connection with an anticancer factor involved. He sets out to show how complexation with cupric ion and oxygen provides an electron-transport chain. There is a firm structure of intuitive reasoning. Then, the dramatic experiment: only the particular derivative found in wheat germ gives the strong color phenomenon, the strong charge-transfer complex. There Kasha, explain that if you can (he implies)! I study some curves previously run by Jane, and think about his structures on paper. Very puzzling. No immediate inspiration springs up in me to supply a quick interpretation. So I am confronted with yet another Szent-Györgyi puzzle to try to unravel, as I have been for the thirty years of our acquaintance.

As I leave Boston Airport by plane I think: what a fortunate man Albert is, and how fortunate would all of us be as scientists to want to rush to the lab on a Sunday morning to do or show yet another novel experiment (at 90!).


Michael Kasha

Michael Kasha was born December 6, 1920, of Ukrainian immigrant parents who had settled in a Ukrainian community in Elizabeth, New Jersey. He received his B.S. Chemistry degree from the University of Michigan (1943) and his Ph.D. (1945) from the University of California.

He was appointed Professor of Physical Chemistry in the nascent Florida State University in September 1951, a position in which he is still actively engaged. In 1960 he founded the Institute of Molecular Biophysics. Since 1962 he has been the Robert O. Lawton Distinguished Professor in the Florida State University. Summer conferences organized by Albert Szent-Györgyi at the MBL in Woods Hole, Massachusetts, influenced Kasha during the decade from 1960 to 1970. He has written on "*Four Great Personalities of Science*—G. N. Lewis, J. Franck, R. S. Mulliken, and A. Szent-Györgyi."

Michael Kasha has worked consistently on research extending the application of molecular electronic theory and spectroscopic interpretation to biomolecular systems. He is a Fellow of the American Academy of Arts and Sciences and a member of the National Academy of Sciences.

Jane A. McLaughlin

Marine Biological Laboratory

Albert Szent-Györgyi's introduction to Woods Hole came in August, 1929, when he attended the 13th International Physiological Congress in Boston. After the meeting, 21 bus-loads of participants came to Woods Hole, as guests of the Marine Biological Laboratory, for a day of science exhibits, demonstrations, and recreation. Before leaving by steamship that evening for New York they were treated to a clambake with lobsters and all the trimmings.

In 1947 when Szent-Györgyi fled Hungary following the Nazi oppression and then that of the Russian occupation, he remembered that summer day of 1929 in Woods Hole. He remembered the laboratory and the delicious lobsters. So, on another August day, eighteen years after his first visit, he and his wife, Marta, turned to Woods Hole for a home. He obtained laboratory space at the MBL—on the third floor of the Lillie Building—overlooking the harbor and there established his Institute for Muscle Research.

Several of Szent-Györgyi's team in Hungary had relocated to other laboratories in Europe; over the next year or so he brought them together in Woods Hole. The first winter there were eight people in the muscle institute. More would arrive later.

Szent-Györgyi divided his time between the National Institutes of Health, in Bethesda, and Woods Hole. His foreign associates at the MBL, whose visas required that they teach, traveled back and forth to New York where they taught twice a week at the New School. Szent-Györgyi was busy trying to raise funds to keep his staff, but most of them had to go elsewhere to make a living. Meanwhile his studies on muscle chemistry were advancing at an impressive rate.

I met "Prof" in the summer of 1952 through Andrew and Eva Szent-Györgyi. I had come to Woods Hole, with a degree in science, from Trinity College of Vermont, looking for a research opening. I was very excited when Prof invited me to work with him. The association continued for thirty-four very interesting years.

That first summer Prof was writing his book *Chemical Physiology of Contraction in Body and Heart Muscle*, which was soon to go to Academic Press. Before I started work at the laboratory I was to read this manuscript, ask questions, and make critical comments. For this I was a house guest at "The Seven Winds"; he and Marta were wonderful hosts. While I probably had questions to ask about the manuscript it is unlikely that this novice found much to criticize. His thoughts were elegantly and simply stated.

I occupied the first floor guest room that also served as a library. During my stay I was being awakened at dawn by noise in the house that I could not identify. After a few days I inquired about what it might be and learned that Prof started typing very early in his study overhead. Later, before breakfast, he would have a swim. When the tide was right he liked to start at the Buzzards Bay beach at the front of his house and swim the swift current that flowed through the Woods Hole channel between the tip of Penzance Point and a nearby island.

Prof would arrive at the lab each morning, a bundle of energy, and could hardly wait to shed his coat before sharing ideas that had sprouted during the night. After a short discussion we would set about doing experiments, sometimes jointly and sometimes each doing a part of it. He was eager to be at his bench, rather than his desk, to be the experimenter and not just the director.

Prof liked quiet. "In laboratories," he said, "delicate instruments are placed on shock-proof tables. I work with my brain so I want that in a shock-proof setting. I found peace and quiet that I needed in Woods Hole and I would not exchange it for millions of dollars with the New York noise."

He did experiments very simply, observed closely, and caught subtle differences. Whether the current idea was concerned with excitation energy, charge transfer, or free radicals, a problem was approached on several fronts. Prof would play with two or three chemicals, the choice having been arrived at overnight, and he would observe the development of some phenomenon, often a color. Further investigations by myself would reveal, more often than not, that he had chosen the optimum conditions for his experiment on the first try. More physical data was accumulated. And because Prof always tried to relate observations to life, to the living system, animal or plant material was then extracted in search of material that was the equivalent of his simple chemical model. Such extracts, natural material, were tested on viable cells or in animals, usually tumor-bearing mice.

Individuals worked on those aspects of the problem that their area of expertise dictated and kept each other advised of developments. Occasionally there would be a seminar, either in the lab or on Prof's south porch.

We usually had a tea break at 4 o'clock. All laboratory members then came together for discussions of general interest, whether scientific, political or cultural. The group was often joined by the few other year-round researchers in the MBL. Organization of the effort for several "causes" often found their beginnings there.

Good spirit was abundant within the institute and that held true for the MBL staff as well. Homer Smith, the general manager, was a welcoming and good host for the Laboratory.

Mrs. Oliver Brown, a contemporary of Prof's, was his secretary for many years. He appreciated how easy she made life for him. When she retired he was more inclined to pen his letters or to type them himself. In the latter case the typing was almost as identifiable as his script. Although readily transcribable, there were abundant errors, with the lines running on and off the page. These are treasured mementoes of Prof.

When someone made a discovery, little or great, a turkey party was held; turkey was chosen because it fed so many people. Invitations went out on the backs of muscle electronmicrographs or the like. Food, drink, music, and dancing comprised the program. At other parties, winter or summer, there was usually a theme, and a costume was a must. Everyone had to work a little to contribute to the success of the affair. On one occasion the institute members were divided into three competitive groups, each was required to present a skit. I don't remember who won the competition, but in one skit Prof played the part of Saint Peter at the Pearly Gates and drew wonderful caricatures of each of us that helped decide our fate.

As Prof approached his seventieth birthday he lost Marta to cancer and was, a few years later, to lose his daughter Nelly to cancer as well. Their final illnesses and deaths were tragic for him.

Prof was usually eager to share his latest find with the world. When he was away from Woods Hole, I often received postcards from him. Their succinct messages tell something about him:

Boston, February 21, 1955 (from Prof and Marta).

"The two youngest USA citizens send their most democratic regards."

Chicago.

"Til now all goes well. I had one fight with Teller. In an hours time will be the second."

Washington, DC, October 11, 1964.

"Regards from Washington just before joining Humphrey for a TV appearance."

Paris, March 4, 1965.

"Paris is wonderful, people vigorous, and in high spirits—which is infective. So I lectured in French and got through alive without a scandal."

Indianapolis, October, 1968.

"East or West, Woods Hole Best."

In the natural course of events, colleagues came and went. Some idea of who they were and what they did may be had by looking at Prof's bibliography, which follows.

During the summer of 1986, just prior to his death when at times Prof was not well enough to come to the lab, we would visit him in his study at home where Marcia, his wife of more than ten years, untiringly cared for him. He was ever eager for the latest results that might give him something new to think about. We would discuss things for awhile and then he would shoo us back to the lab to get on with the work.

Through the years he maintained his good humor, his drive, his enthusiasm, his generous spirit, his principles, his outrage at oppressive situations, his outspokenness about politics, his attitude about constraints on his work, and his concern for the younger generation.

Prof's advice to students reflects the man himself:

> ". . . try to find your real vocation and then work at your vocation with all your heart . . . try to find a corner in this life which you will fill with warmth and light."

Jane A. McLaughlin

Jane A. McLaughlin, a native of Woburn, Massachusetts, a graduate of Trinity College of Vermont, and a corporation member of the Marine Biological Laboratory, has been a resident of Woods Hole since 1952. In addition to numerous scientific papers jointly authored with Albert Szent-Györgyi and others, she has written one book and several articles about Woods Hole history. She was the recipient of the Albert Szent-Györgyi—NFCR 1983 Award for service to science and mankind, on the occasion of Dr. Szent-Györgyi's 90th birthday. She is currently with the laboratory of Dr. Raquel Sussman at the Marine Biological Laboratory.

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Cloning and Aggression among Sea Anemones (Coelenterata: Actiniaria) of the Rocky Shore

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Abstract. Among the common rocky shore anemones of Pacific North America, New Zealand, and Tropical Australia, clonal growth is significantly correlated with other features of the biology including aggressiveness, habitat, and body size. Individual size is more variable among aclonal species and among species living on the lower shore; and aclonal individuals are larger on average than clonal individuals. Aclonal species are usually non-aggressive inhabitants of the lower shore, while clonal species are usually aggressive residents of the upper shore. To explain the link between cloning and aggression, a geometric model is developed that compares the scaling of interference budgets for isometric aclonal growth, and for two-dimensional growth of a compact, encrusting clone. The ratio of exposed perimeter to feeding surface area (P/S) declines more steeply through clonal spread than through aclonal growth; and therefore, the costs of interference relative to energy intake rates should be *lower* for a short, squat clone than for a single, bulbous individual of the same volume. Association with mutually tolerant individuals can also reduce the *per capita* costs of interference (*The Three Musketeers* effect—a special case of the geometric model). Cloning may be more common among anemones living higher on the shore because clonal individuals are generally smaller than their aclonal counterparts, and because predators that prefer small anemones tend to be more abundant downshore.

Introduction

Because they are very different from the kinds of animals usually described as aggressive, the sea anemones (Class: Anthozoa, Order: Actiniaria) provide a potentially useful source of comparative information on the ecology and evolution of intraspecific interference behaviors, and on the evolution of social aggression, which involves both interference and cooperation.

Anemones from rocky shores are soft-bodied, attached, and weakly mobile carnivores that may also derive part of their nourishment from intracellular algae. In addition to growing in a conventional manner when fed, anemones shrink or degrow when undernourished. Some also replicate asexually to form clones of autonomous and mobile individuals; and for these, the maximum size of the clone may be very large, even when individual size is quite small. Furthermore, there is no evidence that anemones necessarily deteriorate with age, though of course many die of natural causes other than senescence (Shick *et al.,* 1979). Consequently, ordinary words like *individual, size, age,* and *growth* can become disconcertingly slippery when applied to the anemones.

Defined for my purposes here, an *individual* (= ramet *sensu* Harper, 1977) is the temporarily distinguishable physical/physiological entity, *i.e.*, a single anemone with a pedal disc for attachment to the substratum, a single opening to the gut for ingestion and egestion, and a set of tentacles surrounding the mouth and oral disc. A *clone* (= genet, *sensu* Harper, 1977) is all of the genetically identical individuals derived from a single zygote by asexual replication. And a *colony* is a group that retains functional connections among the clone members, generally called *polyps* (= modules, *sensu* Harper, 1977).

Aggression is defined conventionally as specialized and directed behavior in which an individual inflicts or attempts to inflict damage on another individual. This is the most active and dramatic form of *interference competition*, which is an activity that's advantage is derived indirectly from its direct cost to a competitor. By contrast, *exploitation competition* is usually understood to be use or monopoly of a resource, an activity that adversely

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effects competitors only incidentally, by limiting their access to resources. *Social aggression* is defined here as active, directed, and more or less coordinated interference between groups of individuals belonging to the same species. While *competition* results in net cost to both parties, *cooperation* is an interaction that results in mutual benefit.

Specialized interference behaviors are known to have evolved only twice among the anemones (Williams, 1975). Some acontiate anemones form long, specialized catch-tentacles that bear large, holotrich nematocysts in place of the nematocyst complement typically found in feeding tentacles (Williams, 1975; Purcell, 1977; Watson and Mariscal, 1983). When these anemones touch a genetically different conspecific (or any other member of the class Anthozoa), one or more of the catch-tentacles expands, extends, and probes the surrounding area. On touching the body of a competitor, the tip adheres and breaks loose from the rest of the tentacle. Some actiniid anemones attack each other using acrorhagi, blunt structures at the top of the column beneath the outer cycle of tentacles (Äbel, 1954; Bonnin, 1964; Francis, 1973b; Bigger, 1976; Brace and Pavey, 1978; Ottaway, 1978; Brace, 1981; Ayre, 1982; Sebens, 1984; Fujii, 1987). When their tentacles touch a genetically different anthozoan, these anemones inflate the adjacent acrorhagi. With stretching and bending movements of the oral disc and column, they wipe these turgid structures against the adversary, leaving large scraps of adhering ectodermal tissue. Firing of the numerous large holotrich nematocysts in the adhering tissue from an acrorhagus or catch-tentacle damages the victim.

These behaviors are specific (Bonnin, 1964; Francis, 1973b; Williams, 1975; Bigger, 1976, 1980; Lubbock, 1980), inducible (Francis, 1976; Purcell, 1977; Watson and Mariscal, 1983) and costly (Francis, 1976). The acrorhagial and catch-tentacle responses serve no apparent functions in feeding or defense against predators, but are elicited only in response to contact with potential competitors belonging to the same or related species. The anemones respond aggressively to contact with genetically different members of their own and other anthozoan species, but passively tolerate contact with genetically identical tissue (their own or that of a clonemate). The specialized weaponry is inducible, so individuals can vary their investment in aggression in response to the particular circumstances.

Isolated individuals and those living in the middle of segregated clones have little or no fighting equipment, while those living in contact with genetically different individuals develop more and larger weaponry and show greatly reduced investment in sexual reproduction. As a further refinement, individuals of some species attack a new neighbor very readily, but are more tolerant of contact with long-term neighbors (Purcell and Kitting, 1982; Sebens, 1984); and some apparently attack only non-clonemates of the same sex (Kaplan, 1983).

Some species of anemones are aggressive and some are not. Since even within aggressive species individuals that are not in contact with competitors apparently reduce or lose their fighting equipment (Hand, 1955b; Williams, 1975; Francis, 1976; Purcell, 1977; Watson and Mariscal, 1983), catch-tentacles and acrorhagi are not likely to become vestigial. I would argue that whatever initially favored the evolution of these inducible interference behaviors, selection must still favor the production, use, and maintenance of this specialized equipment. Of course, the converse is not necessarily true; species living in circumstances favoring the evolution of aggressive behaviors may simply never have developed them. Nonetheless, on average we might expect to find differences in the biology and the ecology of aggressive species, as compared with non-aggressive ones. Data are presented here on the interaction of aggression, individual body size, cloning, and position on the shore; and a simple geometric model is developed to explain the strong correlation between cloning and aggression.

Materials and Methods

Selection of the sample

The sample includes common or locally abundant species from exposed to semiprotected rocky areas where I have worked over the past ten years. Rare species were excluded for consistency, because they were likely to be overlooked in areas where the anemone fauna is not as well described (Southeast Alaska, Baja California, and tropical Australia). Because encounters between members of rare species will generally be uncommon, intraspecific interference presumably will also be uncommon; and eliminating rare species from the sample should simply sharpen the focus of the present study.

Observation and handling of specimens

In little studied areas, I talked with local naturalists, visited accessible rocky sites, and examined the open rocks, crevices, overhangs, and pools at low tide. Each species was observed in the field and, whenever possible, a few individuals were also collected and observed in the laboratory. Specimens of unidentified species were anesthetized with magnesium sulphate (Francis, 1976); squashes of tentacles, and of spherules, warts, and knobs on the upper column were examined microscopically for holotrichs. Anesthetized animals were then preserved in formalin, and those from Mexico and Alaska were later sent to D. Fautin (California Academy of Sciences) for identification.

Study sites

The study areas include two long north-south gradients extending from subarctic to subtropical latitudes, plus a tropical shore. Observations were made and new information is reported (*) from the following sites (arranged north-to-south) along the Pacific Coast of North America: (1) *Torch Bay, Alaska (58°20′ N, 136°05′ W), (2) San Juan Island, Washington (48°28′ N, 123°03′ W), (3) Newport, Oregon (44°40′ N, 124°04′ W), (4) Santa Cruz, California (36°57′ N, 122°04′ W), (5) Gaviota, California (34°27′ N, 120°04′ W), (6) La Jolla, California (32°52′ N, 117°16′ W), (7) *Punta Banda, Baja California, Mexico (MX_1 = 31°44′ N, 116°43′ W), (8) *San Quentin, Baja California (MX_2 = 30°23′ N, 116° W), (9) *Punta San Juanico, Baja California (MX_3 = 26°12′ N, 112°20′ W), (10) *Punta Conejo, Baja California (MX_4 = 24°17′ N, 11°22′ W), and (11) *San Jose del Cabo, Baja California (MX_5 = 23°N, 109°42′ W). Field sites in New Zealand (arranged north-to-south) include beaches on both the east and west coasts of the North Island and the South Island: (1) Leigh, North Island (36°17′ S, 174°48′ E), (2) Muriwai Beach (36°50′ S, 174°25′ E), (3) Abel Tasman, South Island (41°S, 173°E), (4) Kaikura (42°26′ S, 173°44′ E), (5) Okarito (43°14′ S, 170°09′ E), and (6) Portobello (45°51′S, 170°40′ E). Rocky intertidal sites were also examined in North Queensland, Australia: (1) *Lizard Island (AU_1 = 14°69′ S, 145°45′ E), (2) Cape Tribulation (16°04′ S, 145°29′ E), (3) Magnetic Island (19°10′ S, 145°50′ E), and (3) Australia Institute of Marine Science, nr. Townesville (19°15′ S, 147° E).

Assignment to categories

The habitat was categorized either as upper shore (the high and mid-intertidal, extending down to include the zone usually occupied by mussel beds on temperate shores), or as lower shore (the intertidal below mean low tide, and the shallow subtidal just below the lowest tidal excursions).

Sizes reported are individual body size (column diameter in centimeters) for individuals at the upper end of the average reported size range (Parry, 1951, 1952; Hand 1955a, b; Dunn *et al.*, 1980; Bucklin and Hedgecock, 1982; Fautin and Chia, 1986). This was not a search for very large individuals, but rather an estimate of how large individuals ordinarily become.

Species categorized as clonal are those directly observed to fragment, those showing indirect evidence of fragmentation (fission scars), those reported to brood genetically identical offspring, and (tentatively, for species whose growth and reproductive patterns are not well studied) color-variable species occurring in aggregations that are segregated by color-type.

Species were classified as aggressive if even one individual was found with acrorhagi or catch-tentacles. Since these structures are inducible and used only in interference competition, it seems justifiable to use even a single occurrence as diagnostic. Species never observed to produce either catch-tentacles or acrorhagi are tentatively categorized as non-aggressive.

Statistical treatment

A multi-way G-test was used to determine the significance level for shore position, clonal/aclonal growth, and aggressiveness, acting simultaneously. In addition, since some of the theoretically possible sets are empty, it is permissible, if conservative, to use Fisher's exact test with critical alpha levels corrected to compensate for multiple use of data [comparisonwise error rate = $1 - (1 - \text{alpha})^{1/c}$, where c is the number of comparisons and alpha is the desired level of confidence; Sidak, 1967, cited in SAS guide, 1985]. (For three sets of comparisons, a comparisonwise error rate of 0.0165 is equivalent to an experimental error rate of 0.05.)

Development of a model

A geometric model is developed that predicts the relative energy efficiency of interference as a function of increasing size: (1) for growth through isometric increase in size (simulated growth of an aclonal adult), and (2) for growth without increasing in height (simulated growth and spread of a compact, encrusting clone).

Results

Correlations

The individual species included in the sample, together with information on geographic location, shore position, individual size, aggressiveness and mode of growth, are shown in Table I. Simultaneous and pairwise interactions of clonal growth, shore position and aggressiveness are shown in Figure 1 (a three dimensional 2 × 2 × 2 table, projected onto three 2 × 2 tables). Data on individual size are shown superimposed on the same three dimensional matrix (Fig. 2).

Overall, species are not evenly distributed with respect to shore position, clonal/aclonal growth and aggressiveness (Fig. 1) (G-test using a log-linear model with a three-way table, $P \ll .001$). Tested separately, two of the three pairwise interactions are significant (for cloning *vs.* aggression and for cloning *vs.* shore position. $P < .05$ for experiment-wise error rates). Clonal species are more commonly aggressive while aclonal species are more often not aggressive; and clonal species tend to live on the upper shore, while aclonal species are more often found on the lower shore. Using the Fisher's Exact Test (two tailed), comparisonwise error rates are 0.00023 (for

Table I

Individual size, habitat, and habits of common shore anemones from the rocky intertidal of Pacific North America, New Zealand, and tropical Australia, arranged by tidal height

Species**	Clonal	Aggressive	Upper shore[2]	Individual size[3]	Geographic location[4]	Information source[1]
Anthopleura aureoradiata	+?*	+	+*	2	AU, NZ	h, *
Isactinia olivacea	+?*	−	+	3.5	NZ	h, *
Anthopleura sp. 1	+	+	+	1	MX$_{3-5}$	*
Haliplanella lineata (luciae)	+	+	+	2	US	e
Actinia tenebrosa	+	+	+	3*	AU, NZ	h, *
Anthopleura elegantissima (clonal form)	+	+	+	3	AK, US, MX$_{1-4}$*	e*
Cnidopus veratra	+?*	+	+	2.5	AU, NZ	g, *
Anthopleura? sp. 2	+	+	+	3	AU1	*
Phymactis papillosa	+	+	+	3	MX$_4$	*
Aiptasia? sp.	+	−	+	0.5	MX$_5$	*
Phymanthus sp.	−	−	+	5	MX$_5$	*
Phymanthus muscosus	−	−	+	3	AU$_1$	*
Oulactis muscosa	+?*	+	+	4	AU, NZ	h, *
unidentified	+?	−	+	2	AU$_1$	*
Aulactinea incubans	−?	−	+	2.5	US	b
Epiactis thompsoni	−?	−?	0	5	NZ	h
Epiactis prolifera	−	−?	0	4	US	f
Epiactis fernaldi	−?	−?	0	2.5	AK*, US	c, *
Epiactis sp. 1	−?	−?	0	3	AK	*
Epiactis sp. 2	−?	−?	0	3	AK	*
Isocradactis magna	−	+?	0	9	NZ	h
Anthopleura minima	+	+	0	1.5	NZ	h
Anthopleura xanthogrammica	−	+	0	20*	AK, US, MX$_1$*	f, *
Actinodendron sp.	−	−	0	4	AU$_1$	*
Epiactis (Cnidopus) ritteri	−	−	0	5	US	f
Anthopleura elegantissimi (solitary form)	−d	+	0, −*	20*	US, MX$_{1-2}$*	d, f, *
Anthopleura artemisia	+?*	+	0, −*	4	US, AK*	f, *
Metridium senile	+	+	0, −	4	US	e
Urticina (Tealia) crassicornis	−	−	0, −	9	US	f
Urticina (Tealia) coriacea	−	−	−	9	US	f
Actinothoe albocincta	−?	−	−	0.8	AU, NZ	i
Urticina (Tealia) lofotensis	−	−	−	9	US	f
Cricophorus nutrix	−?	−	−	1.5	AU, NZ	i
Phlyctenactis tuberculosa	−	−	−	10	AU, NZ	h
Metridium sp.	−	−	−	10	US, AK	a

[1] = Sources of information: (a) Bucklin and Hedgecock, 1982; (b) Dunn *et al.*, 1980; (c) Fautin and Chia, 1986; (d) Francis, 1979; (e) Hand, 1955a; (f) Hand, 1955b; (g) Ottaway, 1975; (h) Parry, 1951; (i) Parry, 1952; (*) new observation.

[2] = Upper shore animals (+) living at or above the lower mid-tide level; lower shore anemones living near the low water mark (0) and in the shallow subtidal (−).

[3] = Column diameters measured in centimeters for animals at the upper end of reported normal size ranges.

[4] = Glacier Bay, Alaska (AK), Queensland, Australia (AU), Baja California, Mexico (MX), Continental United States, Pacific Coast (US), New Zealand (NZ). Numbers indicate specific sites listed in the text.

** = Species ordered by tidal height, beginning at the top of the shore and working down to the shallow subtidal.

? = Designation tentative.

clonal growth *vs.* aggression and for clonal growth *vs.* shore position), and 0.27 (for aggression *vs.* position on the shore). While it would be desirable to determine whether the factors acting in pairs are effective for a given condition (*e.g.*, the interaction of cloning and aggression within a habitat [upper shore or lower shore]), the samples are not yet large enough to allow meaningful testing.

Overall, the data on individual size (Fig. 2) are not evenly distributed with respect to clonal/aclonal growth (F-test using a linear model, 3-way ANOVA; $P < .05$ both for raw data and for log-transformed data). Clonal species tend to be smaller than aclonal species. Bartlett's test for homogeneity of variance also indicates that the variances are not evenly distributed ($P < .05$). Aclonal species are more variable in body size than clonal species; and those living on the lower shore are more variable

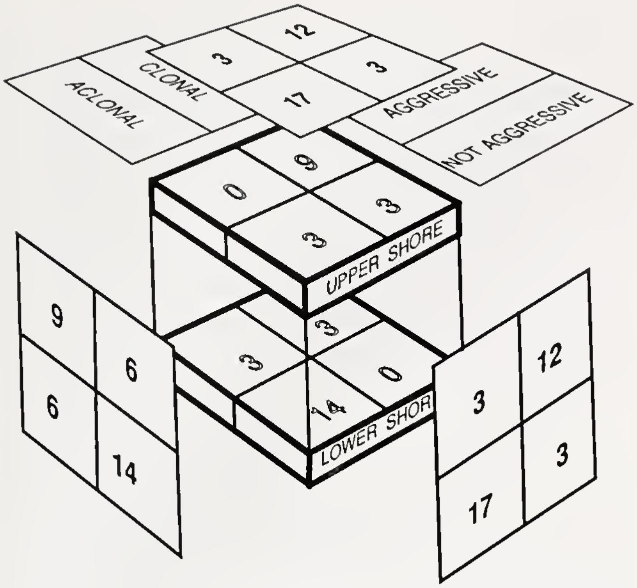

Figure 1. Numbers of common anemone species from the rocky shores of Pacific North America, New Zealand and tropical Australia, grouped by habitat (upper shore or lower shore), intraspecific aggressiveness (aggressive or non-aggressive), and mode of growth (clonal or aclonal). The 2 × 2 × 2 table shows simultaneous interaction among the three factors for the 35 species (center); and the three 2 × 2 tables show interactions for two factors at a time [clonal growth pattern vs. aggression (above), clonal growth vs. shore position (right), and shore position *vs.* aggression (left)].

than those on the upper shore ($P < .05$). The sample is not large enough to reveal any differences between individual cells in the 2 × 2 × 2 table except the most extreme: high, clonal aggressive species are smaller on average than low, aclonal, non-aggressive species, (Tukey-Kramer method for multiple comparisons, $P < .05$ for the experimentwise error rate).

An energy efficiency model: the scaling of interference costs for clonal and aclonal growth

Both the rate of encounter with competitors and the rate of energy intake should vary with the size and shape of the individual, and with the size and shape of the clone (for clonal species). The model developed below predicts the scaling of interference cost as a fraction of the total energy intake for isometric aclonal growth, and for two dimensional growth of a compact clone.

The assumptions of the model are as follows: (1) aclonal growth is assumed to be isometric (no change in shape), as it is for at least some anemone adults (Sebens, 1981). (2) Clones are assumed to form a compact sheet of equal-sized individuals that are attached to the substratum at the base, and that use the upper surface (S) for feeding. (3) Energy intake rate is assumed to increase in direct proportion to the feeding surface area, (Sebens, 1981, 1982a). (4) The rate of contact with competitors, and therefore the rate of expenditure for interference (including cost for making and maintaining fighting equipment, for fighting, and for wound healing), is assumed to increase in direct proportion to the length of the external perimeter (P). (5) Therefore, the fraction of energy intake devoted to interference should be directly proportional to the ratio of exposed perimeter to surface area (P/S).

The basic geometric relationships derived from these assumptions are summarized in equations 1 through 6. For an aclonal individual, the length of the perimeter (P_i) is directly proportional to individual length (L), or to individual volume (V_i) to the ⅓ power.

$$P_i \propto L \propto V_i^{0.33} \qquad (1)$$

Individual feeding surface area (S_i) is proportional to the square of a linear dimension (or individual volume to the ⅔ power).

$$S_i \propto L^2 \propto V_i^{0.67} \qquad (2)$$

Therefore, the ratio of individual perimeter to individual surface area is inversely proportional to length (or individual volume to the ⅓ power).

$$P_i/S_i \propto L^{-1} \propto V_i^{-0.33} \qquad (3)$$

For a compact, clonal sheet, the length of the external perimeter (P_c) is directly proportional to individual length (or individual volume to the ⅓ power), and to the square root of the number of individuals in the clone (n).

$$P_c \propto L \cdot n^{0.5} \propto V_i^{0.33} \cdot n^{0.5} \qquad (4)$$

Clonal feeding surface area (the upper surface = S_c) is

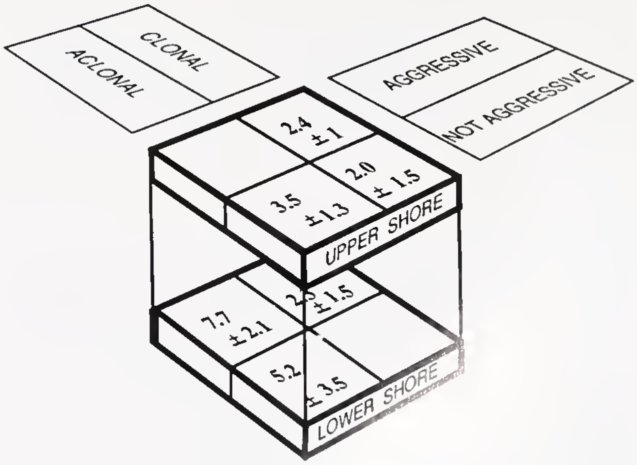

Figure 2. Individual body size as a function of habitat, aggressiveness and clonal growth for 35 species of rocky shore anemones. Figures given are mean column diameter (in centimeters) for individuals at the upper end of reported, average size ranges, plus or minus one standard deviation.

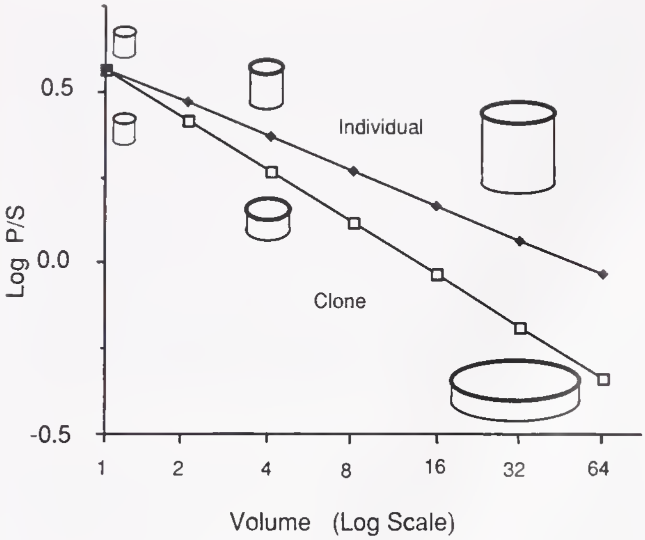

Figure 3. Change in the ratio of external perimeter to upper surface area (P/S) with increasing volume. A. The upper line [$P_i/S_i = 3.69\ V_i^{-0.33}$] shows the decline in P/S with increasing volume for a cylinder that "grows" isometrically, as aclonal anemones do. The following basic, geometric relationships were used in the calculations: L = height = diameter; S_i = upper surface area = $\pi/4 \cdot L^2 = 0.79\ L^2$; P_i = perimeter = $\pi\ L$; $P_i/S_i = 4/L$; V_i = volume = $\pi/4 \cdot L^3$. B. The lower line [$P_c/S_c = 3.69\ V_c^{-0.5}$] shows the decline in P/S for "growth" of a compact, sheet-like clone, modeled as a cylinder that increases in diameter, but not in height. Geometric relationships are as follows: L = height = 1.08; S_c = upper surface area = $n \cdot S_i = \pi/4\ n \cdot L^2$; P_c = perimeter = $\pi\ L \cdot n^{0.5}$; $P_c/S_c = 4\ L^{-1} \cdot n^{-0.5}$; V_c = volume = $V_i \cdot n = \pi/4\ L^3 \cdot n$.

directly proportional to the square of individual length (or individual volume to the ⅔ power) times the number of individuals in the clone.

$$S_c \propto L^2 \cdot n \propto V_i^{0.67} \cdot n \qquad (5)$$

The ratio of clone perimeter to clone surface area is inversely proportional to individual length (or individual volume to the ⅓ power) times the square root of the number of individuals.

$$P_c/S_c \propto L^{-1} \cdot n^{-0.5} \propto V_i^{-0.33} \cdot n^{-0.5} \qquad (6)$$

Predicted differences in the costs of interference relative to energy intake rate are illustrated (Fig. 3) using data for hypothetical cylindrical "anemones" that form short, compact, cylindrical clones. For simplicity, the individual volume of clone members is set equal to one; and clone volume is therefore equal to the number of individuals in the clone (n). Clone feeding surface is also assumed to equal the summed upper surfaces of all clone members [$S_c = n(S_i)$]. Although rigid cylinders cannot be close-packed in this way, the distortions in individual shape necessary to form a compact, cylindrical clone are actually perfectly possible for the protean anemones.

Discussion

Among anemones of the rocky intertidal, the clonal mode of growth is strongly correlated with other important characters including body size, habitat, and aggressiveness. Clonal species tend to be aggressive and to live on the upper shore, while aclonal forms tend to be nonaggressive and to live low on the shore. Twenty-three of the 35 species sampled belong to one or the other of these categories (Fig. 1). *None* are simultaneously clonal, nonaggressive and inhabitants of the lower shore. *None* are aclonal, aggressive inhabitants of the upper shore. This is a very striking pattern that demands explanation.

There are many different methods of asexual replication among the anemones including budding, longitudinal fission, bilateral fission, various kinds of unequal fragmentation (Chia, 1976), and internal brooding of genetically identical young (Black and Johnson, 1979). This suggests that cloning has arisen repeatedly within this group. Further, since clonal and aclonal forms occur within the same genus, and possibly even within the same species (Smith and Potts, 1987; A. Bucklin, pers. comm.), asexual replication may be a relatively labile character that is readily suppressed, as well as repeatedly invented, among the anemones. Therefore, I would argue that the strong correlations between cloning and body size, habitat, and aggressiveness, are ecologically and functionally significant (read "adaptive"), rather than merely historic accidents.

Thoughts on causation: genet size and shape, and the link between cloning and aggression

The geometric model developed here demonstrates the *direction*, not the magnitude, of expected differences in the energy efficiency of interference as a function of size for two different genet shapes.

Where circumstances allow it, growth by cloning should be more advantageous for aggressive species than growth by increase in individual size because the *proportion* of the total energy budget required for interference should be lower for a compact clone than for a large aclonal individual of the same volume ($P_c/S_c < P_i/S_i$, Fig. 3). While increase either in individual size or clone size reduces the ratio P/S, Sebens (1981) has shown that the feeding surface area of anemones increases as a lower power function (of individual weight or volume) than does metabolic rate. Continued increase in *individual* body size beyond the energetic optimum for the particular conditions therefore will result in a progressively lower energy surplus above maintenance level metabolic costs, which eventually will preclude reproduction as well as interference. By contrast, increase in the size of a compact *clone* rapidly reduces the P/S ratio while maintaining an energetically favorable ratio of feeding surface area to volume.

Thoughts on causation: cooperation, kinship, and the link between cloning and aggression

There is an entirely different way to begin thinking about the relationship between cloning and aggression, though it comes to the same thing in the end.

Since the reproductive success of a clone depends on the summed success of its members, a decrease in average *per capita* costs generally increases growth and/or reproductive rates, and increases the fitness of the clone as a whole. However, a behavior that *increases* the costs of a particular individual while causing a greater *total decrease* in the costs (or increase in the benefits) of clonemates would also raise clonal fitness.

The calculus of kinship selection (Hamilton, 1964a, b) was devised specifically to handle this kind of problem. Clonemates are peculiar as kin only because the coefficient of relationship is high and invariant: the fraction of shared alleles is precisely 1.0, or 100%. Among advocates of this approach, it is generally considered good practice to explain phenomena on the basis of individual selection whenever possible, and to invoke kin selection only to explain behavior that consistently *reduces* individual fitness.

In *The Three Musketeers,* Dumas (1844) described reduction of interference costs through *mutual tolerance* and *cooperative association.* Like The Three Musketeers, neighboring clonemates shelter each other from attack on one side. The frequency of aggressive encounters and the individual rate of expenditure on interference will be lower for an individual fighting back-to-back with a clonemate than for a lone fighter of the same size and shape—or more energy can be expended in protecting the exposed sides without increasing total investment. With the same yearly budget for interference, a clonal individual should be able to spend more *per aggressive encounter* than can a single aclonal individual of the same size and shape.

This principle could have broad applicability, since the *necessary* assumptions are few and simple: (1) direct and intense competition with outsiders for some resource, (2) unilateral or reciprocal interference, which is costly both to the initiator and to the recipient, (3) a limited (time/energy) budget for interference, resulting in (4) a (genetically programmed) ceiling on individual investment, and (5) some mechanism for reducing or avoiding interference within the cooperating group.

Kinship within the cooperating group is not necessary. Habituation leading to reduced interference between longterm neighbors (described for *Anthopleura xanthogrammica* by Sebens [1984] and for *Metridium senile* by Purcell and Kitting [1982]) may be examples of *developed tolerance* which decreases interference costs under crowded conditions without sacrificing the ability to discourage newcomers (the dear enemy effect described by Fisher [1954]). *Anthopleura xanthogrammica* is an aclonal, downshore species that lives in very crowded conditions only when it cannot move away from neighbors (Sebens, 1982c). *Metridium senile,* is primarily a resident of the shallow subtidal, where high levels of *interspecific* interference (Sebens, 1985) probably add to its interference costs. It is not yet clear whether these anemones habituate only to contact with members of their own species. The only high shore species tested to date (*Anthopleura elegantissima*) does not seem to habituate at all (N. Withers, cited in Purcell, 1977).

However, cooperation between clonemates *is* unique in being *perfectly stable evolutionarily.* In any other interaction, an individual that can consistently reap the benefits of an association while avoiding attendant costs has a competitive advantage over its purely cooperative neighbor. There is *no* selective advantage to outcompeting a clonemate.

Applied to clonemates, The Three Musketeers analogy can also be formulated as a special case of the energy efficiency model described above. Two additional assumptions are required: (1) Individual size is assumed to be the same for clonal and aclonal genets, and thus individual feeding and metabolic rates can be assumed to be the same. (2) And consequently, clonal genets are assumed to be larger than aclonal genets. These simplifying assumptions probably fit the intertidal anemones well enough to make the model useful for this case. Clones often become very large (Shick and Lamb, 1977; Shick *et al.,* 1979; Sebens, 1982b; Hoffmann, 1986; Fujii, 1987), while individual size in a particular habitat tends to be relatively narrowly constrained by energy considerations (Sebens 1981, 1982a, b) or eventually limited by physiological or mechanical stress.

Since *individual* dimensions are assumed to be constant, clonal feeding surface area is proportional to the number of individuals in a clone.

$$S_c = S_i \cdot n \propto n \qquad (7)$$

Therefore the average length of exposed perimeter per individual (P_c/n) is proportional to the ratio of clonal perimeter to clone surface area.

$$P_c/S_c = P_c/S_i \cdot n \propto \text{[illegible]} \qquad (8)$$

Combining Equations 6 and [illegible] that the average length of exposed perimeter per individual and the ratio of clone perimeter to feeding surface area decline at the same rate with increasing clone size.

$$P_c/n \propto P_c/S_c \propto L^{-1} \cdot n^{-0.5} \propto V_i^{-0.33} \cdot n^{-0.5} \qquad (9)$$

This version of The Three Musketeers analogy (Equation 9) can be reduced to the simple assertion that as the size of a compact clone increases from one individual (= aclonal genet) to greater than one (= clonal genet), the mean interference cost per individual ($\propto P_c/n$) and the fraction of clonal energy intake required for interference ($\propto P_c/S_c$) should both decline at the same rate (shown as the slope of the lower line in Fig. 3).

For species that produce more dispersed or irregular-shaped clones, *per capita* interference costs will be closer to those of an aclonal individual of the same size.

In species that generally produce small clones, most members are in contact with the external perimeter, so interference costs should tend to be quite evenly distributed. Where *actual* individual costs are approximately the same as the calculated average cost per individual, clone fitness will be maximized when investment in interference maximizes *individual* growth plus reproduction.

By contrast, costs can be very unevenly distributed in large compact clones, because members remote from the edge do not engage in interference, while peripheral members commonly have costs far above the calculated average. In species where this is generally the case, selection should tend to favor an individual investment ceiling that maximizes *clonal* growth and reproduction at the *expense* of the individual reproduction and/or growth of peripheral members. For example, clones of *Anthopleura elegantissima* can be very large (Francis, 1973a; Sebens, 1982b); and peripheral members that invest heavily in interference usually do not produce gametes (Francis, 1976). This could be described as kin selection at the level of the clone.

Thoughts on causation: cloning, body size, and habitat

Upshore/downshore differences in both physical and biological selection pressures can be important in determining appropriate body size and shape for sessile marine invertebrates of various kinds (Connell, 1972, 1975; Paine, 1969, 1976; Denny *et al.*, 1985; Jackson, 1977, 1985), including the sea anemones (Johnson and Shick, 1977; Sebens 1977, 1979, 1980, 1981, 1982a, b, d; Francis, 1979; Harris and Howe, 1979; Minasian, 1979; Shick *et al.*, 1979; Annett and Pierotti, 1984; Elliott *et al.*, 1985; Harris, 1986).

Physical forces caused by breaking waves generally prevent growth to large size on the upper shore (Denny *et al.*, 1985); and large size is not energetically feasible for anemones living upshore, where stress levels are high and feeding is limited to relatively short periods during high water (Sebens, 1982a). And indeed, the larger-bodied species in this sample all live on the lower shore.

Coates and Jackson (1985) argue that because cloning allows increase in biomass for a particular genotype without increase in individual (modular) size, aclonal animals should generally be larger than the component modules of related clonal forms; and they cite the British sea anemones as an example. The data presented here confirm and extend that finding. Individual body size tends to be smaller among the clonal anemone species, which more commonly live upshore.

In exposed microhabitats on the upper shore, clones tend to be closely packed and to present a low, streamlined profile (Francis, 1973a). During long periods of low tide, this reduces water loss (Roberts, 1941). Underwater and in steady flows, it also reduces the drag forces experienced by the individuals (Koehl, 1977; Denny *et al.*, 1985). Reduction in interference costs is yet another advantage of clonal aggregation. The tendency for clones to remain together may also favor increased mutual dependence through the development of rudimentary chemical communication (Howe and Sheikh, 1975) and division of labor (Francis, 1976). But aggregation is not without its costs. Uncoordinated asexual replication within clonal aggregations will generally result in severe crowding. Where desiccation, water drag, and interference costs are relatively unimportant, clonal *dispersion* may be advantageous in providing more room for growth (McFadden, 1988; D. Stoner, pers. comm.).

Downshore and in protected habitats where it *is* feasible, large individual size can provide advantages, including the ability to eat larger prey (Sebens, 1981), advantage in competitive interactions (Brace and Pavey, 1978) and better resistance to predators (Sebens, 1977 and 1982d; Harris, 1976 and 1986; Elliott *et al.*, 1985). But in spite of the obvious benefits of unlimited growth through asexual replication, none of the large downshore species are also clonal, suggesting that large body size may be mechanically or strategically incompatible with asexual replication for the anemones. (1) Larger individuals seem to heal very slowly after equal, binary fission (pers. obs.). (2) Fragmentation produces repeated and more or less drastic fluctuations in adult body size. This could be hazardous in the presence of predators, which are generally more abundant and diverse downshore (Paine, 1966; Seed, 1969; Jackson, 1985), and typically prefer smaller prey (Brace and Pavey, 1978; Harris and Howe, 1979; Annett and Pierotti, 1984; Harris, 1986). (3) By internal brooding of small, asexual propagules, a large anemone could avoid significant damage and size decrease while offering protection to the growing clonemate; but again, brooding seems to be linked with small body size for marine invertebrates (Strathmann and Strathmann, 1982).

Among the anemones, growth to large individual size and increase in clone size by asexual replication can be seen as alternative growth strategies (Sebens, 1982a).

Taken together, all of this may explain why small-bodied, clonal species are more common high in the rocky intertidal, while large, aclonal anemones are more common near the low water mark. In exposed positions on wave-swept shores, large individual size is physically impractical. However, through asexual replication, a clone can continue to grow while the component individuals remain small. High on the shore, clonal aggregation can provide some protection both from drag forces and from desiccation stress. Downshore, increasing size-selective predation may generally tip the balance in favor of large, aclonal anemones, even though this usually means hiding in pockets and crevices to reduce hydrodynamic forces, and sacrificing the potential for unlimited (clonal) growth.

Thoughts on causation: habitat and aggression

At a coarse level of resolution, there is an obvious relationship between habitat and the evolution of aggression among sea anemones. (1) Aggression is unknown among burrowing anemones on soft substrata. Exclusive use of a living area may only be worth fighting for if it is relatively stable. (2) On hard substrata, no aggressive species yet described is confined to the subtidal. Some aggressive anemones (*e.g., Metridium senile* and *Anthopleura artemisia*) occur both in the subtidal and in the intertidal, but so far as is known, *none* are exclusively subtidal.

Although a higher proportion of upper shore species in the sample are aggressive (9/15 species or 60%, as compared with 3/20 or 15% of the lower shore species), this difference is not statistically significant.

Aggression among brooding anemones

Information on the genetic relationship between parents and brooded offspring exists for only three brooding anemones. Two species of *Actinia* produce asexual broods (Black and Johnson, 1979; Orr *et al.*, 1982); and both are aggressive toward genetically different individuals and tolerant of neighboring clonemates (Ottaway, 1978; Ayre, 1983; Brace, 1981; Brace *et al.*, 1979). One species of *Epiactis* is known to brood sexual offspring produced through self-fertilization (Bucklin *et al.*, 1984).

If self-fertilization (or inbreeding of any sort) is common among brooding anemones, the coefficient of relatedness might be very high between neighboring individuals. Again, this would tend to stabilize cooperative interactions between neighbors, which could make interference with outsiders more feasible economically. It is intriguing to note that although no aggressive behavior has been reported for members of the brooding *Epiactis* species, they have recently been shown to produce holotrichs (Fautin and Chia, 1986), a type known to function only in aggression, among the actiniarians.

Generalizing beyond anemones: other anthozoans

Volumes have been written on the biology of clonal organisms (Larwood and Rosen, 1979; Jackson *et al.*, 1985; Harper *et al.*, 1986). While a general review is beyond the scope of this paper, it does seem appropriate to comment on some obvious differences between the patterns described here for rocky shore anemones, and what is known about the relationship of cloning to aggression, body size, and habitat among common and closely related animals such as the corallimorpharian anemones, the scleractinian hard corals and the alcyonarian gorgonians and soft corals.

Clonal anthozoans with small polyps certainly are *not* uncommon in the subtidal. The corallimorphs are typically clonal and subtidal. The corals, gorgonians, and soft corals are typically subtidal and *colonial* forms with relatively small polyps. In fact, Jackson (1985) maintains that with the exception of the sea anemones, clonal animals living on hard substrata are generally more abundant and diverse in deeper water than in shallow water.

One important difference between intertidal anemones and the subtidal anthozoans may be vulnerability to predators. Since predator diversities and abundances tend to increase downshore (Paine, 1966; Seed 1969; Jackson, 1985), exposed and sedentary creatures of the shallow subtidal probably require especially effective defenses (Jackson, 1977). The temperate corallimorphs have exceptionally large nematocysts and seem to be less palatable to their downshore predators than are the local Actiniaria (Annett and Pierotti, 1984). Scleractinians secrete a protective hard skeleton; and soft corals commonly sequester toxic chemicals. Furthermore, among colonial organisms, asexual replication need not involve fluctuations in either polyp or colony size. Among colonial forms, resistance to predators may be as much a function of colony size as of polyp size (Jackson, 1985). Parenthetically, the nematocyst-laden acontia of the worldwide, clonal and aggressive anemone *Metridium senile* may be the secret to its success on the lower shore and in the subtidal, since species without acontia seem to be even more vulnerable to predators than is *M. senile* (predator preference tests by Annette and Pierotti [1984] and Harris [1986]).

More generally, then, cloning may [illegible] be associated *either* with low predator pressure (*e.g.*, clonal anemones on the high shore), *or* with powerful antipredator defenses (*e.g.*, the large nematocysts of subtidal and clonal corallimorphs).

Another difference between the intertidal anemones and the typically subtidal anthozoans is that while aggressive anemones will attack nonclonemate members of their own species *and* other species of anthozoans, with a few exceptions (Potts, 1976; Hidaka and Yamazato, 1984), aggression among the subtidal forms is exclusively interspecific (Sheppard, 1982; Chadwick, 1987). Allelopathic chemicals released by some soft corals interfere with the growth of hard coral species (Sammarco *et al.*, 1983) and other soft coral species (Coll and Sammarco, 1986). Some scleractinians and corallimorphs digest neighboring competitors of various species (Lang, 1973; Chadwick, 1987). And some hard corals and gorgonians attack neighbors with specialized and inducible sweeper tentacles (Den Hartog, 1977; Richardson *et al.*, 1979; Wellington, 1980; Chornesky, 1983), or with specialized sweeper polyps (Sheppard, 1982). Although histocompatibility reactions often prevent anastomosis where adjacent coral or alcyonarian colonies come into contact with another clone of the same species (Theodor, 1970; Hildemann *et al.*, 1979), this seems to be an example of growth limitation through exploitation competition, rather than interference.

Again this difference may be related to the difference in habitat. Anemones typically confront relatively few competing species in the intertidal (Francis, 1985); and so direct *intraspecific* competition for space is probably more common and important there than in the subtidal. In the shallow subtidal, where patterns of space use are often more mosaic and changeable (Sebens, 1985), interspecific interference can be quite important, both for the anemones that live there (Chao, 1975; Purcell, 1977; Sebens, 1976, 1985; Shick *et al.*, 1979) and for these other subtidal anthozoans.

So far no one has looked for a correlation between cloning and aggression among the common subtidal anthozoans. The geometric analysis developed for the anemones would require appropriate modifications before it could be applied to three dimensional colonies. However the intuitive version of The Three Musketeers analogy again suggests that interference should be less costly, and consequently more commonly affordable, where tolerant neighbors repel other competitors—regardless of whether the competitor is a member of the same species or of some other species, and regardless of whether the tolerant neighbor is a member of the same colony, clone, or species. Since cooperative association between clonemates *is* unique in being completely stable evolutionarily, *interspecific* interference may also tend to be particularly common among massive colonial forms, and among clonal or colonial forms that ramify or fragment to produce relatively large genets.

However, there are two specific provisos: (1) interference must be costly, and (2) association (with clonemates or other species members) must reduce the costs or increase the benefits of interference. If the corals and corallimorphs that eat neighboring competitors actually *gain* more energy than they lose in the interaction (Sheppard, 1982), then the principle would *not* apply. For alcyonarians, it is also not clear whether the costs of allelopathy can be reduced or the benefits increased through cooperative association.

Like the acrorhagi and catch tentacles of aggressive anemones, the sweeper tentacles and sweeper polyps of some hard corals and gorgonians appear to be specialized, costly, inducible, and locally effective interference structures. For this kind of interspecific interference, costs probably *are* reduced through association with conspecifics; and I would expect these behaviors to be relatively less costly and thus more common or better developed, among species that produce large colonies or individuals among species that commonly form large genets through some kind of asexual replication, and among species that tend to form monospecific aggregations.

The evolution of social aggression

Social aggression implies cooperation within a group of individuals and interference between groups. Therefore, the circumstances favoring the evolution of these behaviors must include *both* those favoring the evolution of intraspecific cooperation *and* those favoring the evolution of intraspecific interference.

The following circumstances seem to favor the evolution of *intraspecific interference* for all sorts of animals, including sea anemones: (1) longevity (no evidence of programmed senescence among anemones), (2) high densities (caused here by local recruitment and accumulation of long-lived, asexually produced individuals), and (3) competition for a limiting resource (in this case, attachment space and access to food).

Circumstances favoring the evolution of intraspecific *cooperation* within an aggressive species include the following: (1) proximity (commonly caused in the anemones by asexual replication), (2) kinship among neighbors (genetic identity among anemone clonemates), and (3) advantage in cooperation (in this case, increased resistance to physical stress for animals in closely packed aggregations, plus reduction of interference costs).

Additional, specific requirements for the evolution of social aggression include the following: (1) cooperative reduction in interference costs or increase in benefits, and (2) the ability to recognize members of the cooperating group and exempt them from attack.

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Foraging Patterns of *Cyphoma gibbosum* on Octocorals: the Roles of Host Choice and Feeding Preference

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Abstract. The distributions of small, relatively slow moving grazers are often portrayed as expressions of feeding preference. However, more careful analysis of such distribution patterns in concert with measurements of feeding suggests that the distribution patterns of the gorgonian-eating snail *Cyphoma gibbosum* may not reflect the influence of feeding preference alone. *C. gibbosum* is common at many sites in the San Blas Islands, Panama, occurring at densities ranging from 2–30 snails/100 m^2. The movements and feeding of 244 labelled snails were followed at three sites in the San Blas during the summers of 1984–1986. Relative to gorgonian colony abundance *C. gibbosum* were preferentially found on colonies of *Pseudopterogorgia* spp., *Pseudoplexaura* spp., and *Plexaura homomalla.* Snails exhibited the same preferences in their movements between colonies, and often remained longer on those same gorgonian species.

The prolonged occupancy on colonies of some species was the dominant factor controlling the amount of tissue consumed from any one species. Thus preferences for different host gorgonians also established total feeding. Preferences loosely correlated with the organic content of the different gorgonian species (% ash-free dry weight), but do not explain the observed preference for *P. homomalla* colonies. Host preferences were not reflected in feeding rates. Feeding rates on *Pseudopterogorgia* colonies were lower than on other frequently occupied species. The weak correspondence between feeding rates and occupancy preferences suggests that factors in addition to feeding such as social interactions and predator avoidance may play an important role in establishing host preferences.

Introduction

Grazers play an important role in controlling the abundance of a wide variety of marine benthic algae and invertebrates (Ogden *et al.*, 1973; Sammarco *et al.*, 1974; Glynn, 1976; Lubchenco, 1978; Hay, 1981; Lubchenco and Gaines, 1981; Lewis, 1986), and thus the preferences of grazers can have important effects on benthic community structure. Recent analyses of prey preferences of marine grazers have emphasized the importance of prey quality, particularly the presence of secondary compounds, in establishing grazer preference (Gerhart, 1984; Steinberg, 1985; Targett *et al.*, 1986, Hay *et al.*, 1987). However, prey choice in many species may not be a simple feeding choice (Hay *et al.*, 1987). In many communities the grazers are small sedentary species that spend prolonged periods of time on single prey individuals. These grazers' prey may serve many functions, and preferential use of some prey species may reflect selective pressures quite different from considerations of feeding alone. It may be more accurate to describe the prey of these species as hosts, and a fundamental question that must be asked in these cases is whether observed host preferences can be adequately described in terms of feeding alone. In this paper we address this question by examining the foraging behavior of the ovulid gastropod *Cyphoma gibbosum* on Caribbean gorgonians.

Since the distribution of *C. gibbosum* on gorgonians is also the distribution of *C. gibbosum* at its feeding sites, *C. gibbosum* distributions have been approached as for-

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aging problems (Birkeland and Gregory, 1975; Harvell and Suchanek, 1987). If considered solely in the context of grazing, gorgonian choice by *C. gibbosum* is analogous to models of which patch a forager should choose and how long it should remain in it (Pyke *et al.*, 1977; Krebs, 1978; Pyke, 1984). However, the patches that *C. gibbosum* uses, *i.e.*, gorgonians, serve as sites for protection, mating, and egg deposition as well as food sources. These behaviors may have a large effect on *C. gibbosum* foraging behavior.

The amount of feeding that occurs on a given gorgonian species is controlled by several potentially independent behaviors: host selection, residence time on the host, and grazing rate. If feeding is the primary factor influencing foraging, then the same gorgonian species would be favored regardless of which behavior is observed. However, movement patterns and the amount of time spent on a colony may also reflect preferences for functions such as mating and egg laying. Therefore, different species could be identified as preferred "prey" depending on which behavior was examined. The extent to which the three different components of foraging identify similar preferences may indicate whether the preferences are best described as preferences for different prey species or simply as host preferences. In characterizing the foraging patterns of *C. gibbosum,* we partition the feeding process into its component behaviors and ask whether species preferences exist and whether they should be termed feeding preferences.

Previous work

A number of researchers have described the activities of *C. gibbosum* (Kinzie, 1970, 1974; Birkeland and Gregory, 1975; Hazlett and Bach, 1982; Harvell and Suchanek, 1987). Although these studies have uniformly verified the specialization of *C. gibbosum* on gorgonians, the differences among reported species preferences are striking. Kinzie (1970) considered *C. gibbosum* to be the principal grazer of gorgonians at Discovery Bay, Jamaica, where for instance, he observed a large *C. gibbosum* aggregation denuding several gorgonian colonies. Kinzie (1970, 1974) did not observe preferences in either the distribution of snails or paired choice experiments.

Birkeland and Gregory (1975) observed the movement and feeding habits of marked *C. gibbosum* during three weeks of intensive study using the Tektite habitat at Lameshur Bay, St. Johns, U. S. Virgin Islands. They report that *C. gibbosum* were found preferentially on *Gorgonia* spp. and *Eunicea succinea,* and that in experiments *C. gibbosum* regularly chose *Gorgonia* spp. over other species. They also found that feeding rates varied with prey species. Feeding rates were greatest among snails on *Gorgonia* spp., but feeding rates were among the lowest on the second most commonly occupied species, *E. succinea.* Harvell and Suchanek (1976) followed marked individuals over a two week period at Salt River Canyon, St. Croix, U. S. Virgin Islands. They found no significant selectivity in the movements of *C. gibbosum,* but they found that snails remained significantly longer on the "*Plexaura* group" (*P. flexuosa, P. homomalla,* and *Pseudoplexaura crucis*) than on *Eunicea* and *Muricea* spp. There were no significant differences in the length of feeding scars generated by snails, but there were significant differences in the depth of scars on different species.

In addition to variation in observed preferences Birkeland and Gregory (1975) and Harvell and Suchanek (1976) report patterns of snail distribution and movement which do not always correlate with observed grazing. This variation underscores the need for longer term multi-site observations which carefully partition the different components of the feeding process.

Materials and Methods

The study was conducted in the San Blas Islands, Panama, at the facilities of the Smithsonian Tropical Research Institute. Observations of *C. gibbosum* feeding were made at three small patch reefs located within several kilometers of San Blas Point. A detailed map of the area is contained in Robertson (1987). The first reef, Macaroon (Porvenir-26 in Robertson, 1987), is located south of a channel through the barrier reef. The reef is 3–4 m deep and is an elliptical hardground approximately 50 m by 30 m which rises 1–2 meters above the surrounding *Thalassia* bed. The reef fauna is dominated by large colonies of the gorgonians *Pseudoplexaura porosa* and *Plexaura flexuosa* and scattered heads of the scleractinians *Diploria strigosa, Siderastrea siderea,* and *Agaricia agaricites.* Gorgonians are the most conspicuous members of the benthos at Macaroon. The area chosen for study was located in the southwest quadrant of the reef.

The second reef, Korbiski (Korbiski-1, SE in Robertson, 1987), is a large reef and reef-flat complex approximately 500 m long and up to 250 m wide. Most of the area is a sand and *Thalassia* reef-flat. The edge of the flat is marked by a narrow band of heavy coral cover (*Agaricia* spp., *Millepora* spp., *Porites furcata,* [illegible]*astrea siderea, Diploria* spp.), which grades into [illegible] slope with scattered head corals (*Montastrea* [illegible] *Montastrea cavernosa, Diploria strigosa, Co*[illegible] *natans*). Gorgonians are common on the slo[illegible]re they are associated with head corals, and [illegible] of the slope where dense aggregations are found [illegible]consolidated coral rubble. The study site was located in an area of mixed hardground and sand 1.0–2.5 m in depth at the northeast tip of the reef. The gorgonian fauna at the site was dominated by *Plexaura* A (see Lasker, 1984, for description of this uncertain species), *Pseudoplexaura porosa, Plexaura homomalla,* and *Plexaura flexuosa.*

The third reef, Pinnacles (Pico Feo-14 in Robertson, 1987), is an area of mixed sand and hardground substrate with scattered clumps of hard coral (*Montastrea annularis, Colpophyllia natans, Porites* spp., *Agaricia* spp.). These clumps, or pinnacles, of coral rise from a depth of 10 m to within 0.5 m of the surface. The gorgonians *Pseudoplexaura porosa, Eunicea* spp. and *Plexaura homomalla* are common on the hard substrate created by the scleractinians. The site chosen for the study was a gently sloping area of sand and coral substrate at 5–7 m depth.

Censuses of *C. gibbosum* were conducted within a single arbitrarily selected 100 m^2 area at each site. The areas were marked and a grid laid at 2 m intervals. All gorgonian colonies within the 10 × 10 m areas were identified to genus and/or species and their heights measured. Colonies which were visited by *Cyphoma* during the study were tagged and their location mapped. Detailed monitoring at the sites was conducted during 1984 (June 8–August 8), 1985 (April 6–June 26), and 1986 (June 7–July 19). During 1984 and 1985 all the sites were searched for *Cyphoma* at approximately three-day intervals. Censuses were conducted weekly during 1986. Newly discovered individuals were removed from the gorgonian colony; the shell scored with a triangular file (after Harvell and Suchanek, 1987); and the snail immediately placed back on the gorgonian. Using a system of markings which ran either perpendicular or parallel to the snail's anterior-posterior axis it was possible to code each shell with a unique mark. At Macaroon, for instance, 61 different individuals were so coded in 1985. The markings were permanent and one individual at Macaroon was observed in each of the three years of observations. The shells of small *Cyphoma* (<1 cm in length) were too thin to score. These individuals were identified on the basis of their size and location. No more than two snails were followed in this manner at any one site.

The distribution patterns of *C. gibbosum* on gorgonians at the different sites were analyzed in a variety of ways. First, the data were analyzed for preference based on the number of times snails were observed on the different species. We call these data occupancy. Second, preferences in choosing colonies were determined by only considering observations in which a snail changed colonies. We call these data movements. Finally the length of each visit to a colony was determined. We call this residence time.

Preferences in occupancy and movements were analyzed with respect to gorgonian abundances using G-tests (Sokal and Rohlf, 1969). In presenting our results we use the ratio of observed to expected occurrences as an index of preference. The observed:expected ratio suffers from a number of shortcomings (Chesson, 1978). However, we use it here because our test statistic G is in large part derived from observed:expected ratios. Indices such as Ivlev's *e* (Ivlev, 1961) use Chi-square statistics, which cannot be partitioned accurately into different factors (Sokal and Rohlf, 1969).

Distributions of *C. gibbosum* on gorgonian colonies were compared to species abundances (Fig. 1). Ideally gorgonian abundance should be measured in a manner that mirrors the snails' ability to find gorgonians and then feed on them. Therefore, abundance could be measured as the number of colonies, their cumulative biomass, or some combination of the two. Since these measures of abundance do not necessarily mirror each other, our estimates of preference could vary depending on the technique used to calculate species abundance. To evaluate the importance of such an effect we analyzed data from one of the sites, Macaroon, using two different indices of gorgonian abundance: the number of colonies, and the sum of the heights of the colonies. The sum of colony heights is an estimator of total biomass. Analysis of the height and total branch length of 1098 5–40-cm tall colonies of three species indicates that height explains 87% of the variation in total colony branch length (Lasker, unpub. data).

The ratio between the number of snails present and that predicted on the basis of host abundance was calculated for each host species using the two indices of host abundance (Table 1). The observed:expected ratios changed markedly when the index of abundance was switched, but there was only a single change in the ranking of host preference. Furthermore, the distributions of snails among the colonies were significantly different from random (G-test) regardless of the abundance index used. As both indices of gorgonian species abundance identified similar preferences, we use numbers of colonies as the index of gorgonian abundance.

The frequency with which the areas were monitored also influences our results. The three-day time interval between samples during the 1984 and 1985 censuses was selected arbitrarily. To determine the degree of bias introduced by this procedure, daily censuses were conducted at Macaroon reef over two four-day periods (April 15–18 and April 20–23, 1985). The total number of moves observed when colonies were censused on a daily basis was 44% greater than that calculated using the censuses from the first and third days. Thus the estimates of movement calculated from the data collected at three day intervals underestimates of the mobility of *C. gibbosum.*

Residence times were compared by analysis of vari-

Table I

Comparison of Cyphoma gibbosum *prey preference at Macaroon Reef during April–June 1985 using two different indices of prey abundance*

	Relative prey abundance		*Cyphoma* observations		
				Preference (observed/predicted) Predicted value based on	
Species	Number of colonies	Summed colony height	% of occurrences	Number of colonies	Summed heights
Plexaura A	0.347	0.237	1.18	0.03	0.05
Plexaura homomalla	0.057	0.063	13.48	2.37	2.13
Plexaura flexuosa	0.206	0.190	5.78	0.28	0.30
Pseudopterogorgia spp.	0.026	0.036	19.70	7.58	5.47
Pseudoplexaura spp.	0.217	0.378	56.74	2.61	1.50
Other	0.145	0.093	3.11	0.21	0.33

ance. Heterogeneity in variances were eliminated using log transforms. As in the movement data the three day sampling interval may have missed short stays. That error was reduced somewhat by the inclusion of single observations as stays of one day.

The amount of grazing on colonies was also monitored during 1985. Damage to branches was measured as linear centimeters of tissue either injured or removed. On planar surfaces, such as colony bases and *Gorgonia* colonies, areal measurements were made. These measurements were converted to cm^3 of damage using species-specific conversion factors based on a set of more detailed measurements which were also collected in 1985 (see below). Damage on each colony was measured each day snails were present, and a final measurement was taken after all snails had left the colony. Net damage was calculated for each time period as the difference between the successive measurements of damage. *Cyphoma* feeding rates were then calculated by adjusting the net damage for the number of snails present. Since it was impossible to distinguish healed areas or overgrown areas from older scars, they were not included in the final damage measurements. Exclusion of healed and overgrown areas should have led to an underestimate of the damage to the gorgonians. In 19.4% of 543 observations, healing and overgrowth lead to observations in which the total scar length decreased between visits. Feeding rates were set to zero in those cases. (Separate analyses in which those cases were excluded yielded slightly higher average feeding rates, but otherwise paralleled analyses including the cases.)

More detailed estimates of feeding rates were made at Korbiski, Macaroon, Pinnacles, and a fourth reef, Sail Rock, during 1985. Colonies with solitary snails were located and the dimensions of the area of exposed axis were measured to the nearest mm with calipers. The area was again measured the following day and the amount of tissue consumed was determined by subtraction. Thickness of the tissue was also measured to convert the areal measurements into volume measures. Observations were excluded from the analysis if the snail left the colony prior to the second measurement or if a second snail appeared on the same feeding scar.

A second set of detailed observations were again made at Korbiski Reef during May and June 1987. In those observations, detailed measurements of the volume of tissue removed were made on the colonies in the 100 m^2 area at three-day intervals. As in the 1985 measurements, rates were calculated by determining the difference in total damage between the successive measurements and dividing by the elapsed time and the number of snails present. All feeding data were analyzed with analyses of variance using log transforms to reduce heteroscedasticity.

The ash-free dry weight of five of the most common gorgonian species was determined from samples of 10 colonies per species. Single branches were collected, allowed to drip dry (2–4 hours), frozen, and subsequently lyophilized. The axes of the dried specimens were then removed and the sample pulverized with a mortar and pestle. The ash weights of samples were determined by ashing five replicate 5 g samples in a muffle furnace for 4 hours at 450°C. Values were converted to unit volume basis using measurements of branch length and diameter which were made *in situ* at the time of collection.

Results

Distribution and movement patterns

The number of snails identified at [illegible] sites, the average number of snails observed [illegible] time, and the total number of observations [illegible] are listed in Table II. Abundances of the most common gorgonian species at the three sites are present[illegible] in Figure 1. Although the abundances of *C. gibbosum* varied between sites and years the movements of snails were very similar. For in-

Table II

Abundance of Cyphoma gibbosum *at three sites in the San Blas Islands, Panama, during the summer months. See text for exact dates*

	Number/100 m^2 mean (standard error)	Total number marked	Observations per individual	Number of days censused	Inter-colony moves per individual	Total number of observations
Macaroon						
1984	21.5 (1.0)	31	10.2 (0.9)	18	3.3 (0.4)	387
1985	22.3 (0.6)	61	11.2 (0.9)	30	3.6 (0.5)	669
1986	27.9 (1.5)	55	2.7 (0.4)	7	1.6 (0.2)	195
Korbiski						
1984	5.1 (0.1)	10	5.2 (1.6)	18	3.6 (0.2)	91
1985	4.5 (0.3)	14	9.5 (2.2)	26	4.0 (0.8)	117
1986	11.3 (0.8)	20	4.1 (0.4)	7	2.5 (0.3)	79
Pinnacles						
1984	7.3 (0.4)	13	9.4 (1.5)	18	3.9 (0.8)	132
1985	16.5 (0.4)	35	11.5 (1.3)	26	5.0 (0.7)	428
1986	2.7 (0.6)	5	—	7	—	19

stance, a given snail could be expected to appear in approximately 43% of a summer's censuses (41, 39, and 48% at Macaroon, Korbiski and Pinnacles respectively). Similarly the incidence of switching colonies between observations was similar between sites (41%, 54%, and 43%, respectively).

Macaroon

Throughout the study, snails at Macaroon exhibited a strong preference in their occupancy patterns on the different gorgonian species (Fig. 2). Although *Pseudoplexaura* spp. were the most commonly occupied gorgonians, when corrected for species abundance the strongest preference was for *Pseudopterogorgia* spp. (Fig. 2). This preference may be attributed to the regular occurrence of a large number of individuals (as many as 12) on a single *P. americana* colony in concert with the low abundance of this species. *Plexaura homomalla* and *Pseudoplexaura* spp. were the next two most frequently preferred species in most years (Fig. 2). In all three years occupancy patterns were significantly different from random (1984, G = 424.8, df = 5, $P < 0.005$; 1985, G = 601.9, df = 5, $P < 0.005$; 1986, G = 423.2, df = 5, $P < 0.005$).

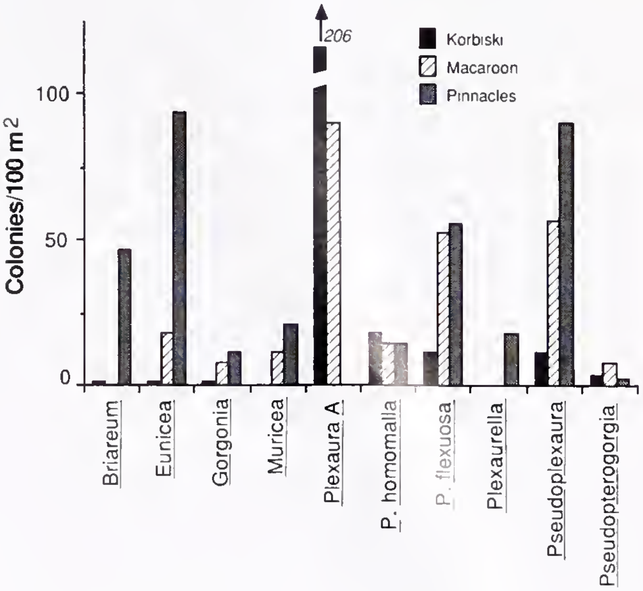

Figure 1. Abundance of gorgonians at three sites in the San Blas Islands, Panama.

As in the occupancy data, *Pseudopterogorgia* spp. were the most preferred species based on moves to colonies (Fig. 2; 1984, G = 24.67, df = 5, $P < 0.001$; 1985, G = 67.23, df = 5, $P < 0.001$; 1986, G = 143.85, df = 4, $P \ll 0.001$). *P. homomalla* was preferred in 1984 and 1986 but not in 1985.

In both 1984 and 1985 occupancy preferences were reflected in the amount of time a snail spent on a colony during a single visit (residence time, Table III). The mean number of days a snail remained on a colony was 9.8 (S.E. = 2.9) in 1984 and 6.9 (0.7) in 1985. Residence times were not calculated for 1986 due to the biasing effect of the seven-day interval between observations. The length of stay during any single visit differed between host species in both 1984 and 1985 (ANOVA of log transformed data; 1984, F = 5.10, df = 5,109, $P < 0.001$; 1985, F = 2.91, df = 5,209, $P < 0.025$). Visits to *Pseudopterogorgia* colonies, the species preferred on the basis of movements, were longer than those on other species. Visits to *P. homomalla* colonies in 1984 were similar in length to those on *Pseudopterogorgia* and greater than on other species.

Korbiski

The occupancy data indicate that *Pseudopterogorgia* spp. and *Pseudoplexaura porosa* were preferred during

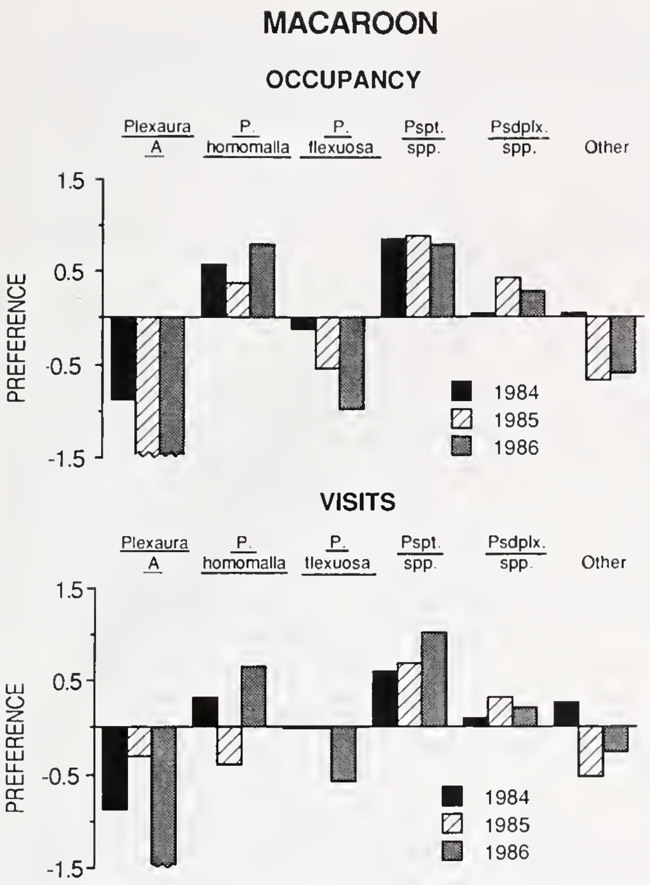

Figure 2. Host preferences of *Cyphoma gibbosum* for different species of gorgonian at Macaroon reef. All values are plotted as the log (observed sightings/expected sightings). Cases of no observation are shown as negative preferences extending beyond the scale. Expected observations are predicted from the relative abundance of the gorgonian species. See text for definitions of occupancy and visits.

1984 and 1985 (Fig. 3; 1984, G = 81.6, df = 4, $P < 0.005$; 1985, G = 383.0, df = 3, $P < 0.005$). *P. homomalla* and a single *Briareum asbestinum* colony were preferred in 1986 (Other category in Fig. 3; G = 242.5, df = 2, $P < 0.005$).

As in the occupancy data, *Plexaura* A—the most abundant gorgonian at Korbiski—was visited less frequently than expected. This strong avoidance of *Plexaura* A colonies was the primary factor creating the significantly non-random movement patterns at Korbiski (Fig. 3; 1984, G = 33.24, df = 3, $P < 0.001$; 1985, G = 49.6, df = 3, $P < 0.001$; 1986, G = 154.38, df = 2, $P \ll 0.001$). Positive preferences in movement patterns were variable between years. The most preferred species in the 1984, 1985, and 1986 seasons were *Eunicea* spp., *Pseudoplexaura* spp., and *P. homomalla*, respectively. The preference for *Eunicea* spp. in 1984 was generated by three short visits to *Eunicea* colonies and is not reflected in the occupancy data. Movement preferences in 1985 and 1986 varied between years but were consistent with the occupancy data.

Differences in residence times on colonies were the dominant factors creating differences in the occupancy patterns at Korbiski. In both 1984 and 1985, the gorgonian colonies on which individual snails remained longest were also identified as preferred species by the occupancy data (Fig. 3). Residence time on *Pseudopterogorgia* and *Pseudoplexaura* spp. were longer than on other species in 1984 (Table III, F = 6.45, df = 5,24, $P < 0.05$). These same two species also had the greatest average residence times in 1985, but differences between species were not significant in the more variable 1985 data (F = 0.98, df = 5,45, $P > 0.25$).

Pinnacles

In both 1984 and 1985, the greatest number of *C. gibbosum* sightings were on *Pseudoplexaura* spp. colonies. However, these sightings were only slightly more common than expected on the basis of *Pseudoplexaura* spp. abundance (Fig. 4; 1984, G = 113.1, df = 3, $P < 0.005$; 1985, G = 179.9, df = 5, $P < 0.005$). Snails were preferentially found on *P. homomalla.* Preferences based on movements to different colonies were virtually identical to those found in the occupancy data. Detailed analyses of the 1986 data were not undertaken because there were only 19 sightings of *C. gibbosum* at Pinnacles in 1986.

Table III

Number of days snails remained on colonies during individual visits

	Length of stay (standard error)		
	Site		
	Macaroon	Korbiski	Pinnacles
1984			
Briareum asbestinum	NA	NA	5.6 (0.9)
Eunicea spp.	NA	NA	4.9 (1.3)
Plexaura A	7.4 (3.2)	1.3 (1.1)	NA
Plexaura homomalla	17.1 (4.8)	0.8 (0.1)	9.1 (3.7)
Plexaura flexuosa	6.1 (1.9)	1.1 (0.2)	18.0 (12.0)
Pseudopterogorgia spp.	23.4 (6.0)	2.7 (0.3)	3.0 (0.0)
Pseudoplexaura spp.	9.6 (2.1)	2.8 (0.3)	NA
Other	5.8 (1.9)	0.7 (0.0)	1.0 (0.0)
1985			
Briareum asbestinum	NA	[illegible]	2.7 (1.0)
Eunicea spp.	NA	[illegible]	5.1 (1.1)
Plexaura A	7.8 (5.5)	[illegible]	NA
Plexaura homomalla	5.4 (0.8)	[illegible]	7.7 (2.4)
Plexaura flexuosa	3.9 (0.9)	[illegible]	3.2 (0.6)
Pseudopterogorgia spp.	12.4 ([illegible])	[illegible] (2.1)	NA
Pseudoplexaura spp.	[illegible]	[illegible].3 (2.8)	5.8 (1.2)
Other	[illegible]	2.2 (0.7)	12.2 (3.6)

NA = species not present or observations lumped in Other category.

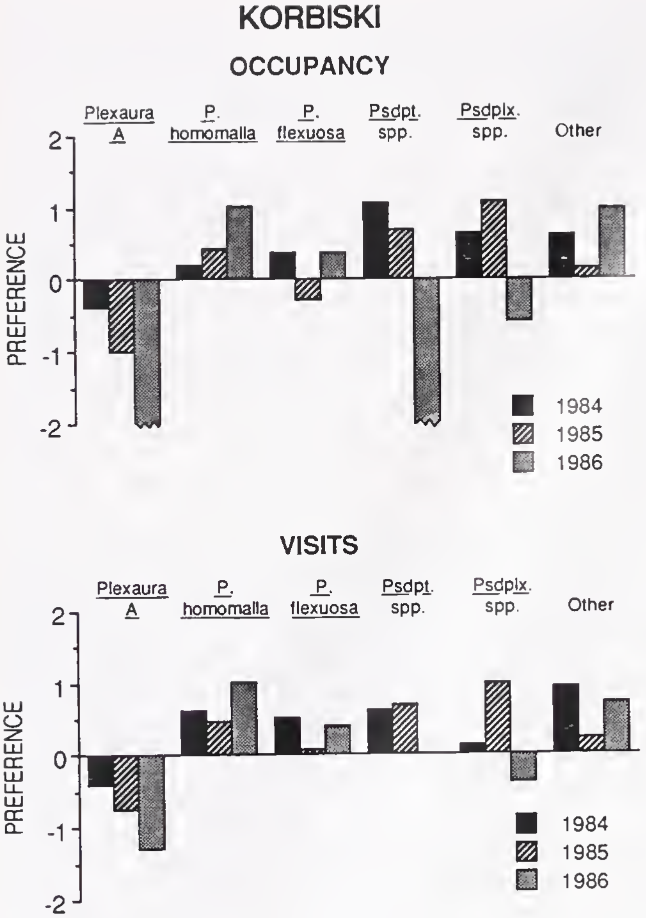

Figure 3. *Cyphoma gibbosum* host preferences at Korbiski. See Figure 2 for explanation.

The average stay per visit during 1984 was 9.0 d (S.E. = 3.0) and was 6.2 d (S.E. = 0.8) in 1985. There were no significant differences among species in the number of days a snail remained on a colony (Table III; 1984—F = 0.72, df = 5,48, $P > 0.50$; 1985—F = 1.57, df = 5,142, $P > 0.1$).

Grazing

The feeding rates measured at the three sites during 1985 were extremely variable (Table IV). Feeding rates on some gorgonian species were an order of magnitude greater at Macaroon than at the other sites. Feeding rates between sites and years differed in the relative ranking of the species as well as in the absolute magnitude of the feeding rate. Feeding rates were greatest on *Pseudoplexaura* spp. at Macaroon, *Plexaura* A at Korbiski, and *P. flexuosa* at Pinnacles. The differences in feeding rates were only significant at Pinnacles (F = 3.86, df = 5,218, $P = .002$), and at that site much of the significant result can be attributed to the absence of measurable feeding on *B. asbestinum* colonies. Aside from the comparisons involving *B. asbestinum* SNK tests indicate that snails at Pinnacles fed at greater rates on *Pseudoplexaura* spp. and on *P. flexuosa* than on "other" species, primarily *Pseudopterogorgia* spp. There were no significant differences between species in feeding rates at Macaroon (F = 2.16, df = 5,265, $P = 0.059$). However, even after transformation feeding rates at Macaroon exhibited significant heteroscedasticity between species (Bartlett-Box F = 4.388, $P < 0.001$).

The most accurate measurements of damage were made in the single day observations of single snails. Four species were compared in those tests, *Pseudoplexaura* spp. (predominantly *P. porosa*), *Plexaura homomalla, P. flexuosa*, and *Pseudopterogorgia americana*. Feeding on the different species differed significantly (F = 5.24, df = 3,115, $P = 0.002$). Significantly lower volumes of tissue were grazed from *Pseudopterogorgia americana* colonies than either *P. homomalla* or *Pseudoplexaura* spp. colonies (SNK test, $P < 0.05$). Rates of feeding on *Pseudopterogorgia* spp. and *Pseudoplexaura* spp. colo-

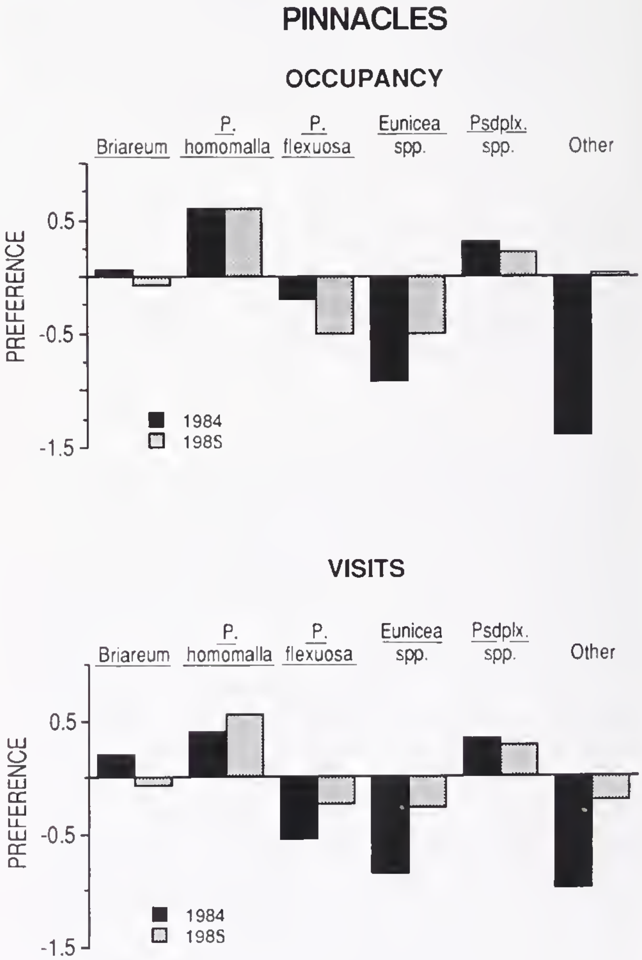

Figure 4. *Cyphoma gibbosum* host preferences at Pinnacles. See Figure 2 for explanation.

Table IV

Rates of feeding of Cyphoma gibbosum *on different species of gorgonians in the San Blas Islands*

	Damage mm^3/Snail/D (standard error)		
	Site		
	Macaroon	Korbiski	Pinnacles
1985			
Briareum asbestinum	NA	NA	8.1 (2.4)
Eunicea spp.	NA	NA	0 (0)
Plexaura A	1.31 (1.31)	10.1 (8.1)	NA
Plexaura homomalla	25.0 (15.9)	2.5 (1.5)	8.4 (2.3)
Plexaura flexuosa	14.6 (5.5)	2.0 (1.1)	14.5 (5.5)
Pseudopterogorgia spp.	40.9 (27.5)	2.0 (1.7)	NA
Pseudoplexaura spp.	343.6 (125.3)	5.3 (1.5)	12.1 (2.1)
Other	54.4 (37.5)	8.1 (8.1)	2.5 (0.9)
1985 Single-day/single-snail observations			
Plexaura homomalla	166.9 (31.4)		
Plexaura flexuosa	199.8 (74.9)		
Pseudoplexaura porosa	277.4 (42.5)		
Pseudopterogorgia americana	81.5 (13.7)		
1987 Korbiski observations			
Plexaura A	168.7 (49.6)		
Plexaura homomalla	137.0 (26.7)		
Plexaura flexuosa	288.2 (46.4)		
Pseudoplexaura porosa	129.7 (47.4)		
Pseudopterogorgia americana	114.7 (56.7)		

NA = species not present or observations lumped in Other category.
Different techniques for assessing damage where employed in 1984 and 1985. See text for method used to estimate feeding rates.

nies were somewhat similar to the measures made at three-day intervals at Macaroon, but most of the single-day/single snail rates were much higher than those made at three-day intervals in 1985.

At first glance, differences between the single-day and three-day interval measurements made during 1985 suggest that healing and overgrowth may have biased the three day interval observations. However, the detailed measurements of feeding scars conducted in 1987 yielded results similar to the single-day observations even though the 1987 measurements were taken at three-day intervals and involved as many as 11 snails on a single colony. This suggests that the lower values of the other 1985 data sets may be attributable to the more rapid but less accurate technique of extrapolating volumes consumed from measurements of feeding scar lengths. Feeding rates on *P. flexuosa* colonies were greater than those on either *Pseudopterogorgia* spp. or on *Pseudoplexaura* spp. (ANOVA, F = 4.33, df = 4,125, $P < 0.003$ and SNK tests $P < 0.05$). In both data sets involving detailed measurements of the volume of tissue consumed, feeding on *Pseudopterogorgia* spp. colonies was lower than that on other colonies.

Table V lists the ash-free dry weight of equal volumes of tissue of five common gorgonian species. *Pseudopterogorgia americana* and *P. porosa* contained the highest levels of organic matter per unit volume.

Discussion

At the sites in the San Blas, *Pseudoplexaura* spp. (usually *P. porosa*), *Plexaura homomalla*, and *Pseudopterogorgia* spp. (usually *P. americana*) were disproportionately occupied by *C. gibbosum*. Were those patterns indicative of preferences for different species and, if so, were they feeding preferences? We examine these questions in greater detail by breaking the foraging process into its component parts: movement, residence time, and grazing. In each we consider whether sampling errors or social behaviors may have produced patterns which could incorrectly be interpreted as species preferences. We have previously concluded that *C. gibbosum* distribution patterns are affected by such biases (Lasker and Coffroth, 1988). In this analysis we note such biasing effects and conclude that species preferences also contribute to the distribution patterns. We then consider whether the preferences are also feeding preferences.

Table V

Ash-free dry weight content of five common gorgonians from the San Blas Islands

	Ash-free Dry Weight (mg/mm^3)			% Ash-free Dry Weight	
	Mean	Standard deviation	n	Mean	Standard deviation
Plexaura A	0.17	0.04	10	39.6	11.9
Plexaura flexuosa	0.10	0.02	10	15.9	4.2
Plexaura homomalla	0.09	0.06	9	37.7	6.9
Pseudoplexaura porosa	0.20	0.06	10	73.8	6.6
Pseudopterogorgia americana	0.30	0.03	10	73.7	9.0

Movement patterns

The first step in establishing host choice is movement to a colony. Foraging snails can follow one of three general patterns: they may wander randomly until they locate and occupy a colony; they may randomly locate a colony but then occupy it on the basis of some trait of the colony; or they may locate and occupy a colony on the basis of either their own history or the presence or absence of other snails.

The data from San Blas strongly indicate that movement patterns are not random. However, the observed pattern could arise if snails moved randomly but sampled and rejected some colonies on a time scale smaller than the interval between observations. In this case the preferred colonies on which snails remained longer would act as "traps" for the snails. We would find an apparent trend for more moves to some colonies even though the moves had occurred randomly. Sampling at three-day intervals missed as many as 44% of the moves noted in daily monitoring. However, we do not believe the three-day sampling scheme biased the preferences in movements, because the colonies that contained snails in the daily sampling were no different than those identified in the three-day sampling. If our sampling procedure masked random movements, it probably did so by missing cases in which snails left colonies within minutes or hours of their arrival. In observations of snails crawling on the substrate we observed snails to reach a colony, "taste" it, and then move on. Similarly, we observed colonies on which we never found *C. gibbosum* but which exhibited linear patterns of small *C. gibbosum* "tasting" scars (see also Gerhart, 1986). The absence of these sightings in our data set may bias it against finding snails on colonies that were rapidly abandoned. Therefore, the movements reported should be defined as visits, where visits are stays of one or more days. The data clearly demonstrate that *C. gibbosum* visits were not distributed randomly among colonies in the 10 × 10 m sites.

Apparent preferences would also be generated by the presence of snails with home ranges or territories, a phenomena suggested by Ghislein and Wilson (1966). However, our data do not exhibit the patterns of visits that would be expected in a territorial species. If *C. gibbosum* had territories, those snails with territories entirely within the study area should have been observed more often than those snails whose territories only partially overlapped the study area. Thus one would expect to most commonly find rarely appearing snails at the periphery of our areas. To test for this we examined the data for snails that were seen only once, and we compared the proportion of those observations occurring within the central 6 × 6 m of the area (36 m^2) to those occurring within the outer 2 m of the area (64 m^2). During the 1985 observations at Pinnacles, 13 of 15 single observations occurred within 2 m of the edge ($G = 3.91$, $P < 0.05$). However, no significant edge effect was observed in the other five data sets (G-tests, all values $P > 0.05$). Some snails appeared to confine their movements to small groups of colonies, but these snails occupied colonies throughout the area, and a snail that was restricted to a small area during one sequence of censuses often appeared at the other end of the 10 × 10 area in the next census. These observations do not exclude the possibility that territories exist, but they indicate that territoriality did not have a great effect on the patterns that we observed.

Apparent species preferences could also occur if snails selected only large colonies and if large colonies were restricted to only a few species. To determine whether the species preferences that we observed were in fact size preferences, we compared the size frequency distribution of the colonies visited to that of all colonies at the site. Since some gorgonian species are consistently smaller than others, analyses combining all species compound size effects with species effects. Therefore, separate analyses for size preferences were conducted for each species for which the colonies covered a range of sizes. When small colonies were present, there were cases of visits and feeding on colonies of all sizes. No significant size preferences were detected among *Pseudoplexaura porosa* and

Eunicea spp. colonies at Pinnacles, nor were size preferences detected among either *P. porosa* or *Plexaura flexuosa* colonies at Macaroon. Larger colonies of *Plexaura* A were preferred at Macaroon. This may reflect the fact that the *Plexaura* A population contained a number of quite small (<30 cm) colonies and these colonies were never the sites of extended visits. Aside from the absence of aggregations on very small colonies there was no relationship between size and the location of *C. gibbosum* aggregations on these species.

Finally *C. gibbosum* may not have been locating specific colonies but rather the presence of other snails. *C. gibbosum* exhibited aggregated distributions at the San Blas Is. sites (Lasker and Coffroth, 1988). Gerhart (1986) reported that *C. gibbosum* recognizes and follows the mucous trails left by other individuals. He proposed that this behavior might explain aggregated *C. gibbosum* distributions. The aggregation of individuals on colonies could exaggerate preferences and possibly generate an appearance of a preference for a species that had initially been chosen at random. Stochastic effects in concert with aggregative behavior undoubtedly contributed to the patterns observed in the San Blas (Lasker and Coffroth, 1988).

Although social interactions affect the distribution patterns in the San Blas, the data also demonstrate the existence of true species preferences. If the observed distributions of *C. gibbosum* were generated by snails following mucous trails and not by species preferences, then at any one time each site should have had several colonies on which groups of snails were present. If there were no species preferences, the location of the aggregations should have changed randomly over time as old trails decayed and "lost" snails located new colonies. Colonies on which more than three snails occurred changed between years. However, the same species were consistently occupied during the different years. During the three summers, 25 different colonies were identified as holding more than three snails. These aggregations lasted for at least two observations and in 1985 one colony contained three or more snails on 18 of the 30 census days. The colonies on which aggregations most commonly occurred were *Pseudoplexaura* (12 colonies), *P. homomalla* (7), and *Pseudopterogorgia* colonies (2). *P. homomalla* and *Pseudopterogorgia* spp. were used in great excess relative to their abundance. *P. porosa* colonies were also used more often than expected, but *P. porosa* usage more closely mirrored local abundance (see also Lasker and Coffroth, 1988).

Residence time

The next component of *C. gibbosum* host preferences is the amount of time a snail remains on the colony, or residence time. Our observations missed extremely short visits. The absence of these very short visits to "non-preferred" species should have biased our data against finding differences in residence times on different colonies. Despite this inherent bias, we observed significant differences in residence time between host species in three out of the six data sets. In those cases where significant differences in residence time were observed, stays on species identified as preferred by the occupancy data were significantly longer than stays on other species. A somewhat similar pattern also was observed among the nonsignificant data sets (Table III).

Gerhart (1986) reports that the length of time *C. gibbosum* individuals remain on *P. homomalla* colonies is reduced if the colony was previously occupied. Our data, which pool observations on many different species, exhibit the opposite trend. The probability of a snail leaving a colony was calculated by considering each observation of a snail on a colony as a replicate case. Cases were partitioned between colonies with only one snail present and those with more than one snail. The probability of leaving a colony was not positively associated with the presence of other snails (G-test, $P > 0.05$ in 8 data sets - Korbiski and Macaroon 1984, 1985, 1986, Pinnacles 1984, 1985). During 1985 the year with the greatest number of observations a significant positive association was found between the presence of other snails and remaining on a colony (G- tests, $P < 0.025$, all three sites). Colonies on which individuals remained for long periods of time were also the colonies that the most snails visited. This pattern was repeated at all of the sites in all years. At each site there was at least one colony that snails frequently visited, and on which they invariably remained for extended periods. In several cases these colonies were sites for egg deposition or were occupied by juveniles (snails <1 cm length). The colonies with the longest history of occupancy by *C. gibbosum* and on which *C. gibbosum* had the longest residence times were invariably *P. porosa* and *P. americana*. The most "popular" of these colonies, a *P. americana* at Macaroon, has contained groups of 2–12 snails for 7 years. Although our results differ from Gerhart's (1986), his study only examined *P. homomalla*, whereas our data include observations from other species. Unfortunately our data contained too few observations of snails on *P. homomalla* to directly test Gerhart's experimental results.

Grazing rates

The final component of the feeding process is the grazing rate. As we have already discussed some of the variance in the 1985 observations made at three-day rates may be measurement error. However, detailed measurements made in the 1985 single-snail/single-day observations and again in 1987 are also characterized by tremendous variability. Much of this variability can be attributed to the fact that gorgonians are not only feeding sites, and that much of the time spent on a colony is not used for feeding. We frequently observed snails laying eggs, mating, or simply "sitting" in the middle of feeding scars. The effect of social behaviors is suggested by the presence of a significant *negative* correlation between the feeding rate on a colony and the number of snails present (Korbiski, 1987, $r = -0.18$, $n = 130$, $P = 0.019$).

The one trend appearing consistently was the lower feeding rates observed on *Pseudopterogorgia* colonies. If the volume consumed was dependent on the number of "bites" taken or the time spent actively feeding, these data suggest an aversion for *Pseudopterogorgia* as a food source. Thus the data on feeding rates only partially agree with the distribution patterns.

The rate at which *C. gibbosum* feed in conjunction with the amount of time a snail spends on a colony defines the total amount of tissue that a snail ingests. *Plexaura homomalla, Pseudoplexaura porosa,* and *Pseudopterogorgia americana* were the most commonly occupied gorgonians, and therefore *C. gibbosum* fed more often on these species than other species. If the trends in feeding rates are also considered then we can tentatively conclude that *Pseudoplexaura* spp. and *P. homomalla* made up the greatest proportion of the *C. gibbosum* diet.

What drives Cyphoma gibbosum *host preferences?*

The movement and residence preferences create a pattern of feeding in which the species preferred on the basis of occupancy make up the greatest proportion of the *C. gibbosum* diet. Therefore we will first consider the hypothesis that the movement and feeding patterns of *C. gibbosum* are dietary preferences based on prey quality.

Quality of gorgonians as prey species can be divided into two categories: nutritive content and defensive quality. Nutritive content in turn can be divided into the absolute food content of the gorgonian and the different types of compounds present. When expressed as a percentage of the ash free dry weight of the tissue there are not large differences in the protein, lipid, and carbohydrate content of the different gorgonian species (Lasker, unpub. data). However, there are large differences in the sclerite content of the different species. Thus, the greatest difference in the nutritive value of the different species is in the organic content of the tissue. *Pseudopterogorgia americana* and *P. porosa,* the species with the highest levels of organic matter per unit volume (Table V), were preferentially occupied by *C. gibbosum.* Even after adjusting for the lower feeding rates on *P. americana, C. gibbosum* would have a higher rate of organic intake on these species than on the other gorgonian species. Furthermore, feeding on these two species would allow snails to obtain a given amount of nutrition in the smallest amount of time. Thus *Pseudopterogorgia americana* and *Pseudoplexaura* spp. should be preferred if *C. gibbosum* foraged in a manner that either maximized organic intake or minimized foraging time. However, *P. homomalla*—the other frequently occupied species—had the lowest average organic content, and would be a poor food choice under either of the foraging criteria. Organic content may play a role in establishing preferences, but it does not explain all of the observed pattern.

The low levels of feeding on *Plexaura* A and *P. flexuosa* also suggests that sclerites could act as a deterrent to feeding by *C. gibbosum.* Harvell and Suchanek (1987) suggest the presence of such a relationship in the feeding rates that they measured. However, in the San Blas, *P. homomalla* was eaten regularly despite its high sclerite content (sclerites make up the major portion of the ash component of gorgonian tissue). Furthermore, feeding rates on *P. flexuosa*—the species with the highest ash content—were high in 1987. Again, there is some correspondence between feeding and sclerite content, but our data indicate that sclerite content alone is a poor predictor of *C. gibbosum* foraging behavior.

The presence of chemical defenses within the gorgonians also fails to explain the observed feeding preferences. Many authors have commented on the presence of secondary compounds in gorgonians (Ciereszko and Karns, 1973; Tursch *et al.,* 1978; Lee *et al.,* 1981; Fenical, 1982; Gerhart, 1984), and both *P. homomalla* and *P. porosa* contain toxic compounds. *P. homomalla* contains impressive quantities of the prostaglandin PGA_2 (Schneider *et al.,* 1977; Gerhart, 1984, 1986), and *P. porosa* contains crassin acetate (Lee *et al.,* 1981). Both of these compounds have toxic effects on organisms (PGA_2—Gerhart, 1986; crassin acetate—Perkins and Ciereszko, 1973; Lee *et al.,* 1981). Although we do not know what compounds may be in the other gorgonian species, it is clear that *C. gibbosum* is neither picking its prey nor adjusting its feeding rate to avoid the toxin in these two species. If chemical defenses affect *C. gibbosum* feeding, then additional factors such as the snail's resistance to given defenses must also be considered.

There is some correspondence between the occupancy patterns and the nutritive quality of their gorgonian prey. However, the observed preference for *P. homomalla* colonies as well as the inconsistencies in observed feeding rates suggest that additional factors affect *C. gibbosum* movement and residence patterns. As alternatives to feeding preferences we consider the hypotheses that *C. gibbosum* choose gorgonians on the basis of their suitability as sites for egg laying and/or as sites for predator avoidance.

C. gibbosum deposit their eggs on the bare axis of gorgonians, which are exposed by the snails' feeding activities. At some of the sites the colonies chosen for egg laying paralleled the occupancy preferences, but the trends in egg laying were poor predictors of occupancy preferences. Egg laying was observed on *Plexaura homomalla, Pseudoplexaura porosa, Pseudopterogorgia americana, Plexaura flexuosa,* and a *Eunicea* sp. At Macaroon eggs were most frequently deposited on *P. porosa* colonies (13 of 16 observations of newly laid eggs)—a species also preferred based on the occupancy data. However, at Pinnacles, a site in which *Pseudoplexaura* spp. were preferentially occupied, those species were never used for egg deposition. Similarly, *Pseudopterogorgia americana* was frequently occupied at Macaroon but was only used for egg deposition on one occasion. *Eunicea* spp. were most commonly used for egg deposition at both Pinnacles and Korbiski, but overall host use mirrored egg laying on *Eunicea* spp. in only one instance, Korbiski, 1986. The small number of snails engaged in egg laying at the three sites (no more than 4 each at Pinnacles and Korbiski and 16 at Macaroon) makes it difficult to determine with confidence whether distinct egg laying preferences exist. However, the data indicate that the distribution patterns observed were not driven by preferences for sites of egg deposition.

Another hypothesis that could explain movement patterns is based on the protection different host species offer. Predation is an important source of mortality in the San Blas. Mortality averaged 18.3%/year at Macaroon and 6.8%/year at Pinnacles (Lasker and Coffroth, 1988). Shell debris from mortality events suggests that crabs, stomatopods, and fishes are responsible for 60% of the mortality.

The largest and most stable aggregations of *C. gibbosum* occurred on *P. americana* colonies. This species was only used for egg deposition once and was grazed at a lower rate than other gorgonians. *P. americana* colonies are large and structurally complex, and *C. gibbosum* located in *Pseudopterogorgia* colonies are difficult to find. This may protect snails from visual predators such as fishes. Higher survival might also account for our frequent observations of small snails on these colonies.

Predator avoidance might also be the basis for the occupancy of *P. homomalla* and *Pseudoplexaura* spp. colonies. Gerhart (1986) showed that fish find the mantle of *C. gibbosum* distasteful, and suggested that *C. gibbosum* coloration and aggregations warn potential predators of the presence of toxic compounds which the snail has obtained from its food sources. Schneider (1972, in Steudler *et al.,* 1977) isolated the prostaglandin PGB_2 from *C. gibbosum* tissue and suggested that the gorgonian prostaglandin PGA_2 was a likely precursor of this secondary compound. The *C. gibbosum* host preferences observed in the present study are clearly compatible with Gerhart's hypothesis.

The distribution of *C. gibbosum* on its gorgonian prey exhibits a complex pattern reflecting the snail's use of the gorgonian as both prey and host. We found no correlation between known chemical defenses and *C. gibbosum* feeding, but the preferential use of some gorgonian species correlated with their organic content/structural defenses. This suggests that food quality plays a role in determining *C. gibbosum* distribution patterns. However, the poor correspondence between distribution patterns and feeding rates suggests that additional factors play as great a role as feeding in establishing *C. gibbosum* foraging behavior. Additional factors that may explain some aspects of the snail's distributions should be explored in future research. These are: the possible protective role of the host colony, the social interactions which generate the aggregative behavior, and intraspecific variation between gorgonian colonies which could explain why only a small subset of gorgonian colonies serve as aggregation sites.

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An *In Vitro* Analysis of Egg Mortality in *Cancer anthonyi:* The Role of Symbionts and Temperature

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Abstract. Several symbionts occur on crab eggs. These symbionts, and the effect of temperature, have been implicated as causal mechanisms of egg mortality in *Cancer magister.* The contribution of three symbionts (a fungus, *Lagenidium callinectes;* bacteria, *i.e., Leucothrix* sp.; and a nemertean worm, *Carcinonemertes epialti*) to egg mortality on *Cancer anthonyi* were investigated *in vitro* using a multifactorial experimental design at four different temperatures. The nemertean worm was found to contribute most to egg mortality on the individual crab and at the crab population level because its prevalence was high (>95%) and it had a relatively constant feeding rate. *Lagenidium callinectes* caused from 20–75% egg mortality on individual crabs but its prevalence was nil. A *Lagenidium*-like fungus had a low prevalence (2.6%) and was not associated with egg mortality. While bacteria were omnipresent, they were found to cause negligible crab egg mortality. Few significant interactions were observed between the symbionts. Temperature had a significant effect on worm feeding rates, worm oviposition, and fungal attack rates. At low temperatures (4 and 10°C), symbionts killed fewer eggs than at higher temperatures (15 and 20°C). Extreme temperatures (4 and 20°C) caused variable degrees of egg mortality, yet some eggs survived at these temperatures. Temperature also had a profound effect on egg development. At 20°C, eggs developed almost twice as fast as those at 10°C. Development appeared to stop at 4°C.

Introduction

Several symbionts have recently been reported to cause egg mortality in the broods of the Dungeness crab, *Cancer magister.* A nemertean worm, *Carcinonemertes errans,* eats the eggs of *C. magister* (Wickham, 1978, 1979a, 1980; Roe, 1984). At high densities, it may account for the high incidence of egg mortality observed on *C. magister* (Wickham, 1979a, 1980). Other symbionts have also been implicated as possible agents of crab egg mortality. Bacterial fouling (*e.g., Leucothrix* sp.) of the egg masses of crabs is highly correlated with worm number, developmental stage of the crab eggs, and egg mortality (Fisher, 1976; Fisher and Wickham, 1976; Wickham, 1979a). The fungal pathogen, *Lagenidium callinectes,* has been reported in cultures of larval *Cancer magister* (Armstrong *et al.,* 1976), but its presence in the clutch of *C. magister* has not been well established. The prevalence of *L. callinectes* on the eggs of the blue crab, *Callinectes sapidus,* is correlated with the prevalence of *Carcinonemertes carcinophila* (Rogers-Talbert, 1948).

Nemertean worms, various fungi, and numerous bacteria may all be found on the same crab (Fisher and Wickham, 1976; Shields, pers. obs.). Therefore, it has been difficult to determine if the symbionts act separately, or in conjunction, to produce egg mortality. Hamilton (1984) showed that crab egg mortality may result from either fungal infection or nemertean infestation. However, fungi were present in her egg cultures containing *Carcinonemertes errans.* Fisher (1976) experimentally increased bacterial fouling by increasing the available nutrients present in seawater. In control treatments with antibiotics, egg mortality decreased. However, *C. errans* may have been present in the broods of these experimental crabs (Wickham, 1979a). The contribution of the other symbionts to brood loss has not been examined.

The cyclic fluctuations in *Cancer magister* populations have received much empirical and theoretical attention (*e.g.,* Botsford and Wickham, 1978; Wild, 1983;

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Hankin, 1985). High temperature (Mayer, 1973; Wild, 1983) and nemertean egg predation (Wickham, 1979a, b) have been invoked as possible contributory factors in the decline and non-recovery of the central California population of *C. magister.* While temperature variation clearly influences the rate of egg development and egg mortality, the role of *Carcinonemertes errans* in producing egg mortality has been incompletely assessed (Wild, 1983).

To date there have been no quantitative studies of crab eggs exposed to only *L. callinectes,* bacteria (*Leucothrix* sp.), or *Carcinonemertes* species. We used a multifactorial experimental design to determine the relative contributions *in vitro* of each symbiont (*Carcinonemertes epialti, Lagenidium callinectes,* and *Leucothrix* sp.) to the egg mortality of *Cancer anthonyi. Cancer anthonyi* was used throughout this study as it is locally abundant and breeds year-round (Reilly, 1987). The susceptibility of eggs in different developmental stages was also examined. In addition, the effect of temperature on egg mortality and development, and on symbiont behavior was investigated.

Materials and Methods

Ovigerous crabs were collected at nearshore depths of 10–100 m by a commercial fisherman. Crabs were trapped from the Santa Barbara Channel, between Summerland and Gaviota, California, and maintained at ambient seawater temperatures (12–17°C) in 280 l flow-through fiberglass aquaria. Twenty-five crabs were used in the study.

A single ovigerous pleopod was excised from crabs bearing embryos in various stages of development. The developmental stages of embryogenesis (EDS-egg developmental stage) were assessed as per Shields (1987). Egg-bearing setae were removed from the pleopod and placed in UV-filtered seawater (2–35 μm filters, one ultraviolet-light filter, Rainbow Plastics, Filter Division, El Monte, California). Samples consisting of 80–250 eggs attached to individual and intertwined setae were counted while using a dissecting microscope, and the number of dead eggs and their apparent cause of death (*e.g.,* mechanical disruption, infertility, etc.) were noted. After counting, the sample was washed in UV-filtered seawater containing 1.0% bleach for 2–4 minutes to kill or remove microorganisms, and placed in a 35 × 10 mm plastic petri dish with 3.0 ml of UV-filtered seawater containing, in some treatments, antibiotics [500 mg each of penicillin-G and streptomycin sulfate per liter (Sigma Co.)]. The egg samples were then exposed to various pathogens detailed below and were placed in an incubator at 4, 10, 15, or 20°C. Five to ten replicates of egg samples were examined in each treatment.

Various egg samples were exposed to male or female *Carcinonemertes epialti.* Worms were removed from the excised pleopods of crabs by vigorous agitation in 200–300 ml seawater. Worms were taken from crabs whose eggs were in similar stages of development to those in the experimental treatments. Individual worms were then pipetted into a petri dish where they were sexed prior to being placed with the previously counted egg samples. Egg predation by the worms, worm behavior in the petri dish, and the number and time to hatching of worm egg strings were noted every two days for the duration of each experiment. Egg production was measured as the mean number of egg strings produced by each female worm at each stage of crab egg development. The egg predation rate was determined for each worm by dividing the number of eggs eaten by ten—the number of days in each experiment.

A pure culture of *Lagenidium callinectes* was acquired from the American Type Culture Collection (#24973 ATCC). Cultures of *L. callinectes* were grown in liquid Kazama's modified Vishniac medium (KMV: 1.0 g glucose, 1.0 g gelatin hydrolysate, 0.1 g bacto-peptone, 0.1 g yeast extract, 1.0 l seawater, 1.0 g agar). Cultures were maintained both with and without antibiotics (500 mg each of penicillin-G and streptomycin sulfate per liter) at 15 and 20°C. Zoospores and resting spores from 2- to 6-week-old cultures of *L. callinectes* were counted using a hemocytometer (Levy counting chamber). Three replicates of the culture were counted and the appropriate dilutions were made to give an estimated density of 150 zoospores/ml. Various egg samples were then exposed to 1.0 ml of the diluted *L. callinectes* culture. To examine the effect of initial zoospore density on egg mortality, exposures of 15, 150, and 1500 zoospores/ml were examined at 15°C.

The prevalence of *L. callinectes* from wild *Cancer anthonyi* was examined. Isolation of *L. callinectes* was attempted for monthly or bimonthly samples of *C. anthonyi* eggs. Egg samples were removed from crabs and placed in sterile petri dishes containing UV-filtered seawater. Fresh samples were examined for the presence or absence of bacteria and fungi. After three days, the samples were again observed for symbionts. Using sterile pipettes, the samples were then streaked onto sterile agar (KMV with antibiotics) petri dishes. Seawater controls were also cultured. After an additional 3–5 days, the plates were examined for fungal symbionts.

Cultures of *Leucothrix* sp. and other bacteria were isolated on KMV from crab egg clutches. No attempt was made to isolate individual species of bacteria. The cultures were used only if *Leucothrix* sp. was present. Filamentous bacteria and the typical "fingerprint" colonies of *Leucothrix* sp. (Johnson *et al.,* 1971) were used to indicate the presence of usable, experimental cultures.

Counts of bacteria were made in the same manner as the fungal counts. A density of 75,000 bacteria/ml was used in the study. Egg samples were inoculated in the same manner as the those exposed to the fungus.

Egg samples were exposed to each symbiont alone and to each combination of symbionts. A separate treatment of eggs exposed to antibiotics and diluted KMV medium was a control for the use of these products. An additional treatment of eggs that were not exposed to bleach, antibiotics, and KMV, was an additional control for the various manipulations. Crab egg mortality was assessed every two or three days for ten days.

Statistical procedures are described in Sokal and Rohlf (1981). Arcsin transformations were used when appropriate, *i.e.*, to normalize ratios and percentages. A value of $P < 0.05$ was significant.

Results

General observations

Eggs killed by worms were either wholly or partially devoid of yolk material; their egg coats were torn. Eggs killed by the fungus became opaque. Infected embryonic tissue was characterized by black and brown spots of pigment. Infected eggs initially appeared fuzzy. Later, fungal exit tubes protruded from the surface of the eggs. In later stages of development, embryos killed by the fungus were often misshapen, and roughly ellipsoid in form. Such an appearance presumably resulted from reduced internal pressure upon zoospore penetration. Misshapen eggs appeared to have an intact internal coat surrounding the embryo. Eggs killed by bacteria eventually became opaque and possessed a filamentous border.

At each temperature, some eggs were developmentally retarded. Many of these eggs never developed further. The cause of this phenomenon is unknown. Data on asynchronous development of the embryos are presented below.

Carcinonemertes epialti

No significant differences were detected between egg predation rates for worms in the presence or absence of bacteria (worms alone, and worms with bacteria). These treatments were, therefore, combined for the analysis. The feeding rates for both sexes were combined for those treatments maintained at 4°C since little egg predation occurred at that temperature. Only 10 of the 75 worms examined at 4°C ate eggs.

The predation rate of male *C. epialti* varied with temperature. Male worms had a significantly lower predation rate at 4 and 10°C than at the higher temperatures (Fig. 1A: ANOVA, Sidak's inequality, $P < 0.05$). However, there were no significant differences between predation rates for treatments at 15 and 20°C (ANOVA, Sidak's inequality, $P > 0.05$). The predation rates of male worms were not significantly different between the stages of crab egg development within each temperature regime.

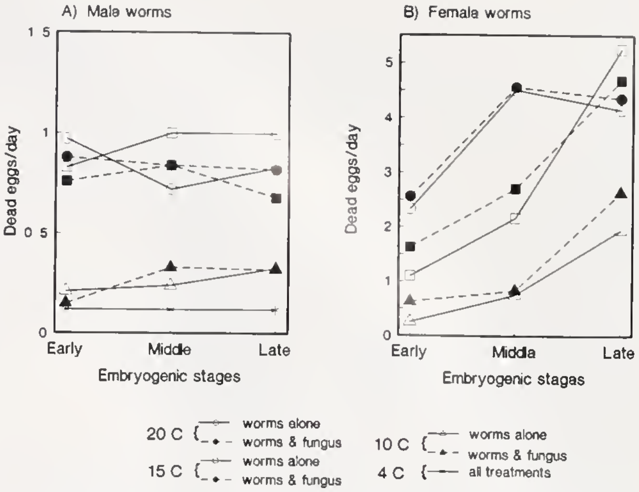

Figure 1. Predation rates of *Carcinonemertes epialti* feeding on the eggs of *Cancer anthonyi* at different temperatures. Early, middle, and late refer to stages in crab egg development. (A) Predation rates of male worms; (B) Predation rates of female worms. N ≥ 5 replicates in all treatments.

Female worms had a much higher predation rate than male worms (Fig. 1B). The predation rate of female worms increased with temperature and with the developmental stages of the crab eggs (Fig. 1B). At 4 and 10°C, the predation rate at each stage of egg development was significantly less than those rates at the higher temperatures (ANOVA, Sidak's inequality, $P < 0.05$). Female worms fed at significantly lower rates during the early and middle stages of egg development at 10 and 15°C, than during the latter part of embryogenesis (ANOVA, Sidak's inequality, $P < 0.05$). At 20°C, however, predation rates were highest during the middle of embryogenesis. They were significantly greater than the predation rate in early embryogenesis (ANOVA, Sidak's inequality, $P < 0.05$).

Egg production by *C. epialti* varied with temperature and crab embryogenesis (Fig. 2). The total production of egg strings was significantly lower at 4°C than at the other temperatures (ANOVA, Sidak's inequality, $P < 0.05$). There were no significant differences in total egg production at 10, 15, and 20°C temperature regimes. However, significant differences were found in the timing of egg production between these temperatures (Fig. 2). At 10°C, females produced egg strings while feeding on eggs in the limb bud stage of development (EDS III, Shields, 1987). At 10 and 15°C, egg production and the number of reproducing females increased with crab egg development. At

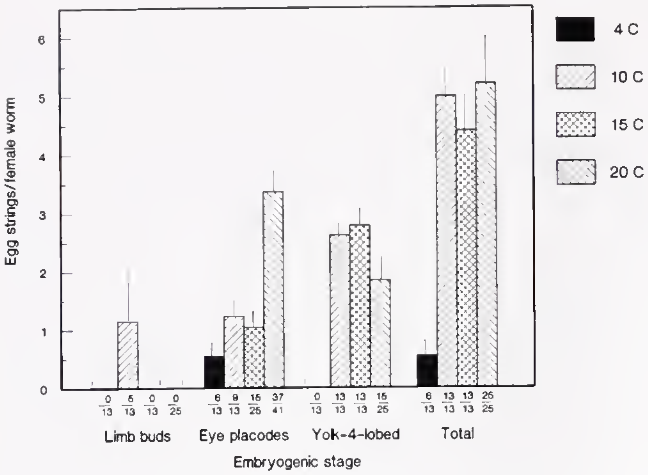

Figure 2. Egg production, measured as the number of egg strings produced per female *Carcinonemertes epialti*, in relation to temperature and crab embryogenesis. Numbers refer to egg-laying females and total number of females. Bars represent standard errors.

20°C, egg production and the number of reproducing females were highest in the eye placode stage of crab egg development (EDS IV, Shields, 1987), but these decreased significantly (ANOVA, Sidak's inequality, $P < 0.05$) in the four-lobed yolk stage (EDS VI, Shields, 1987).

Overall predation rates by the nemertean worms were not affected by the presence or absence of *L. callinectes*. However, changes in worm behavior were noted in those treatments containing the fungus. In general, when egg mortality was associated with the presence of the fungus, the worms no longer resided on the eggs or setae. Most often, the worms secreted new mucous sheaths directly onto the surface of the petri dish; yet some worms persisted on the infected eggs. Egg predation dropped only as a result of massive egg mortality caused by the fungus. *Carcinonemertes epialti* never ate eggs infected with *L. callinectes*, whereas eggs infested with bacteria were often preyed upon by the worm.

Lagenidium callinectes

Growth and attack rates of *L. callinectes* were affected by temperature and the different stages of egg development (Table I, Figs. 3, 4). At 4°C, no fungal growth was observed for any treatment during the experiment. At 10°C, *L. callinectes* had extremely low attack rates throughout the developmental period. The fungus infected significantly more eggs at 15°C than at 10°C, and infected significantly more at 20°C than at 15°C (ANOVA, Sidak's inequality, $P < 0.05$). Significantly fewer eggs were attacked during early embryogenesis than during middle and late embryogenesis at 15°C (Table I, Fig. 3: ANOVA, Sidak's inequality, $P < 0.05$).

The growth of *L. callinectes* was positively associated with initial zoospore density (Fig. 4). At low and moderate zoospore densities (15 and 150 zoospores/ml), the fungus attacked few eggs; subclinical infections were common (see below). At the high zoospore density (1500 zoospores/ml), fungal attack rates were significantly higher. These results must be interpreted with caution as the variances between treatments were not equal.

Temperature had a profound effect on the establishment and attack rates of the zoospores from the high initial density (Fig. 4). Significantly more dead eggs were found at 20°C than at 15°C in those replicates exposed to 1500 zoospores/ml ($T = 2.71$, $P < 0.02$).

Typically, *L. callinectes* grew either well or poorly in each replicate. The marked variation in the ability of the fungus to attack and kill eggs is shown by the large standard deviation and standard error in most treatments (Table I, Figs. 3, 4). Subclinical infections of *L. callinectes*, defined as infections not presenting fungal exit tubes and with few hyphae present, were observed in many replicates. These low level infections contributed to the high variation in attack rates. Subclinical infections killed few eggs during the ten-day trials. However, in longer trials (14+ days) these infections often became acute and produced higher death rates.

Lagenidium callinectes was never actually observed on any of the *Cancer anthonyi* collected in the Santa Barbara Channel. A *Lagenidium*-like fungus was observed in a few samples of crab eggs taken over the course of one year. The *Lagenidium*-like fungus had an overall prevalence of 2.6% (6/232 crab clutches). The fungus was found in three broods with eggs in early stages of development, two broods with eggs in mid or late stages of development, and one brood with eggs at eclosion. The fungus was not associated with high levels of crab egg mortality. Attempts to isolate this fungus failed.

No significant interactions were observed between the fungus and the bacteria. *Lagenidium callinectes* grew and attacked eggs at approximately the same rate regardless of the presence or absence of *Carcinonemertes epialti* or bacteria (Table I). However, *L. callinectes* often thrived in the presence of *C. epialti*. Fungal zoospores frequently attached to mucus produced by the worm and hyphae were occasionally noted on worm sheaths. Fungal growth was also noted frequently on crab eggs that had been damaged during the establishment of the experiment.

Bacteria

Bacteria were always present to some extent in the broods of *Cancer anthonyi*. Bacteria were never observed at high densities on the eggs of *C. anthonyi*. Egg mortality in the treatments containing bacteria and in control

Table I

Mean number of infected crab eggs (±SD) after ten days exposure to Lagenidium callinectes *at different temperatures*

		Developmental stages of the eggs		
Temperature	Treatment	Early	Middle	Late
10°C	F	2.6 ± 2.4 (5)	1.0 ± 1.7 (5)	0.0 (5)
	FW	0.8 ± 0.8 (9)	5.9 ± 3.7 (9)	0.4 ± 0.7 (9)
	FB	0.6 ± 0.9 (5)	1.6 ± 1.5 (5)	2.6 ± 3.2 (5)
15°C	F	3.7 ± 9.3 (20)	29.8 ± 35.9 (20)	32.6 ± 44.0 (20)
	FW	3.0 ± 2.8 (25)	12.0 ± 20.0 (29)	11.1 ± 16.3 (29)
	FB	6.0 ± 29.9 (5)	11.8 ± 15.7 (5)	30.2 ± 36.3 (5)
20°C	F	94.6 ± 32.8 (10)	79.2 ± 54.0 (9)	131.6 ± 75.2 (10)
	FW	125.9 ± 60.5 (9)	39.4 ± 48.6 (9)	129.5 ± 87.4 (9)
	FB	Not done	Not done	Not done

Treatments include presence of fungus alone (F); fungus and *Carcinonemertes epialti* (FW); and fungus and bacteria (FB). Numbers in parentheses represent the number of replicates per treatment. At 15°C, mortality in early stages of development was significantly less than mortality in later stages (ANOVA, Sidak's inequality, $P < 0.05$).

treatments was extremely low (Table II), and was not influenced by temperature.

Temperature and other sources of mortality

In some cases, egg mortality occurred in the absence of observed symbionts. In particular, temperature shock may have caused significant mortality (Table III, and see below). At 4°C, many eggs had swollen outer coats. By the end of the experiment, these eggs had died. Temperature shock may have also contributed to mortalities at 20°C. Asynchronous egg development was most apparent at 20°C (Table III) and to a lesser extent at 15°C. Asynchronous development was most notable in treatments containing eggs in late stages of development. Typically, some eggs developed normally, whereas other eggs developed at a much slower rate. Ovigerous crabs held at 15°C possessed few eggs in asynchronous development. Thus, experimental conditions may have contributed to the observed asynchronous development.

Temperature had a marked effect on embryogenesis of *Cancer anthonyi* (Table III; Fig. 5). Development proceeded more quickly at higher temperatures (15 and 20°C) than at lower temperatures. At 4°C, no visible egg development occurred. Interestingly, eggs held at 4°C for 10 days and then returned to 15°C renewed normal development. The duration of each developmental stage

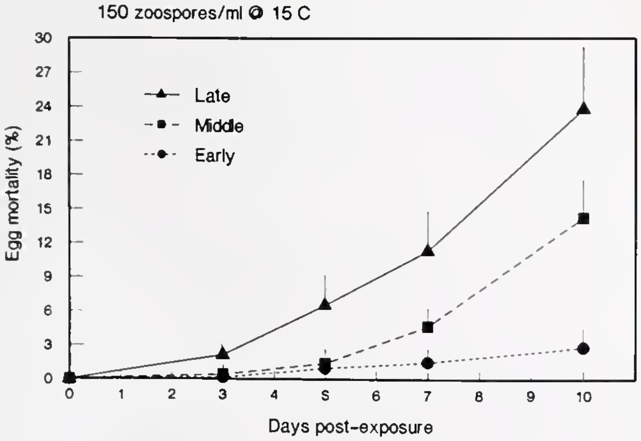

Figure 3. Growth of *Lagenidium callinectes* on different developmental stages of *Cancer anthonyi* eggs at 15°C. Growth was measured as a function of egg mortality. Twenty replicates per treatment. Bars represent standard errors.

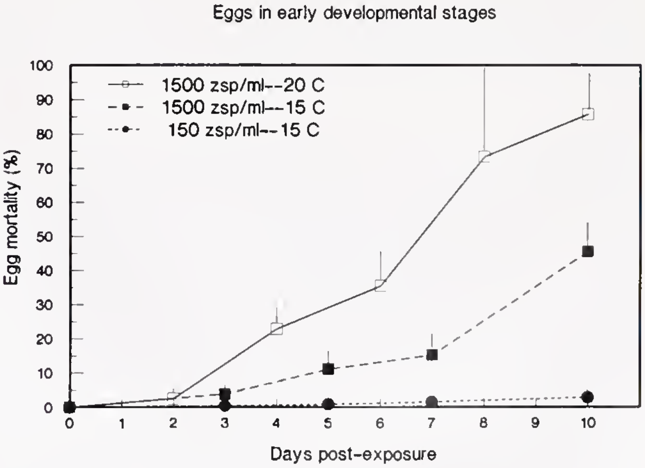

Figure 4. Growth of *Lagenidium callinectes* at different zoospore densities on the eggs of *Cancer anthonyi*. Twenty replicates per treatment, except for the 20°C treatment (5 replicates). Bars represent standard errors.

Table II

Mortality rates after ten days for bacterial treatments (B) and for the control treatments (C-untreated control, C_{aa}-bleach, antibiotics and agar control)

		Developmental stages of the eggs		
Temperature	Treatment	Early	Middle	Late
10°C	B	0.6 ± 0.7 (10)	Not done	0.5 ± 0.7 (10)
	C_{aa}	0.4 ± 0.5 (5)	0.0 (5)	0.0 (5)
	C	0.0 (5)	0.0 (5)	0.4 ± 0.5 (5)
15°C	B	0.6 ± 0.9 (5)	0.0 (5)	0.0 (5)
	C_{aa}	0.6 ± 1.0 (10)	0.7 ± 1.1 (10)	0.4 ± 1.0 (10)
	C	0.8 ± 0.6 (10)	0.1 ± 0.3 (10)	0.1 ± 0.3 (10)
20°C	B	0.6 ± 1.3 (5)	0.2 ± 0.4 (5)	0.0 (5)
	C_{aa}	0.5 ± 0.7 (5)	0.6 ± 0.8 (10)	0.1 ± 0.3 (10)
	C	0.4 ± 0.9 (5)	0.4 ± 0.8 (10)	0.5 ± 0.7 (10)

Numbers in parentheses represent replicates per treatment. There were no significant differences between treatments within temperatures.

became proportionally accelerated with increasing temperature (Fig. 5). At 20°C, considerable variation was noticed in the rate of development of eggs from different clutches. Accelerated development, wherein eggs developed at a much faster rate, was evident in some replicates at this temperature. It occurred primarily in eggs in late developmental stages, especially in eggs progressing from the eye placode stage to the yolk band stage (EDS IV–VI, Shields, 1987).

A protistan fungus, *Rhizophydium littoreum* (Chytridiomycetes), was isolated from field samples and some experimental treatments. Experimental exposures *in vitro* indicated that although the chytrid was associated with dead eggs, it was not a causal mortality factor (Shields, unpub. data).

Discussion

This study implicates *Carcinonemertes epialti* as the primary cause of egg mortality of *Cancer anthonyi*. In the presence and absence of other symbionts, these worms caused substantial egg mortality *in vitro*. The high prevalence (>97% of the ovigerous crabs were infested, Shields, 1987) and the relatively high predation rates of female worms at various temperatures suggest that these egg predators contribute most to egg mortality on individual crabs.

Outbreaks of nemertean egg predators on *Cancer magister* (Wickham, 1979a, b), *Hemigrapsus oregonensis* (Shields and Kuris, 1988), *Homarus americanus* (Aiken

Table III

Observations on the effects of temperature on egg mortality and abnormal or asynchronous development of Cancer anthonyi *embryos*

	Temperature related mortality			Asynchronous development		
Temperature	Early	Middle	Late	Early	Middle	Late
4°C	++	++	++	No development		
10°C	—	+	—	—	—	—
15°C	—	—	—	±	±	±
20°C	++	±	+	++	++	++

Key to mortality and development symbols: ++, present in large proportion of replicates, and to large degree in individual replicates; +, present in small proportion of replicates; ±, infrequently observed in replicates; —, not observed in replicates.

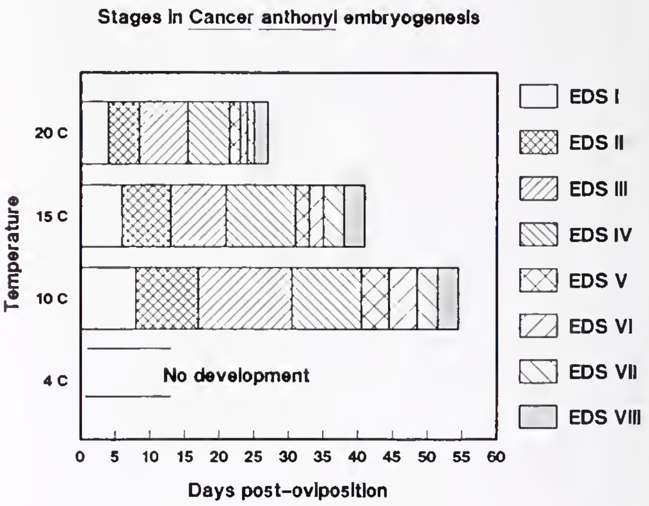

Figure 5. Development of *Cancer anthonyi* eggs as a function of temperature. EDS represents the developmental stage of the egg as per Shields (1987). EDS I represents eggs in one-cell stage through blastulation; EDS II, gastrulation through development of tagmata; EDS III, limb buds evident through buds well developed; EDS IV, development of eye placodes and eyes, faint heartbeat in live eggs; EDS V, completed eyes, yolk in 4 distinct lobes, strong heartbeat in live eggs; EDS VI, yolk reduced to a band or two lobes; EDS VII, yolk reduced to two small masses; EDS VIII, hatching. Data from composite results.

et al., 1985; Campbell and Brattey, 1985), and *Paralithodes camtschatica* (Wickham *et al.*, 1985, and in prep.) are associated with massive host egg mortality. The impact of these outbreaks on the reproduction of the individual host and host populations may be quite high (Wickham, 1979a, b; Wickham *et al.*, 1985; Kuris and Wickham, 1987; Shields and Kuris, 1988). While *Carcinonemertes epialti* has never been found at high, outbreak densities on *Cancer anthonyi*, it is clearly capable of causing the moderate levels of egg mortality observed on that host species.

Predation rates and aspects of reproduction of *Carcinonemertes* spp. have been examined (Wickham, 1979a, 1980; Roe, 1984; Kuris and Wickham, 1987). The feeding rates of mature male and female *C. epialti* at 15°C agreed with those observed by Roe (1984) at 12–14°C (males—1.3 eggs/day, females—2.4 eggs/day). No significant differences were reported between the predation rates of *C. epialti* and *C. errans* (Roe, 1984). Egg production—the total number of egg strings produced—averaged 3.1 egg strings/female for *C. errans* on *Cancer magister* (Wickham, 1980), 2.8 and 5.6 egg strings/female for *C. epialti* on *Hemigrapsus oregonensis* (Roe, 1979, 1984, respectively), and approximately 5.0 egg strings/female for *C. epialti* on *Cancer anthonyi* (this study). The predation rate of both *C. epialti* and *C. errans* increases with the onset of worm maturation and reproduction, and is closely timed with the development of the crab eggs (Roe, 1979; Wickham, 1979a, 1980; Shields 1987). This pattern was not affected by temperatures above 10°C. Worms ate and matured faster at higher temperatures, but total egg production was not significantly greater at elevated temperatures.

The timing of egg production by *Carcinonemertes epialti* was affected by temperature. The faster onset of reproduction in females at 10°C compared to that at higher temperatures may be explained by the timing of crab embryogenesis. Crab eggs developed faster at higher temperatures, hence worm reproduction occurred on eggs in later stages of development. Since the initiation of oviposition by *C. epialti* is closely tuned to crab embryogenesis (Roe, 1979; this study), and since crab embryogenesis may be greatly affected by temperature (Fig. 5), then the faster onset of reproduction and egg laying by *C. epialti* at 10°C may enable larval worms to hatch prior to and during host eclosion. Timing is important because female *Cancer anthonyi* strip the empty egg shells and associated debris (including worm egg strings) from the pleopods within 48 hours of crab egg eclosion (Shields and Kuris, in prep.).

In the present study, *Lagenidium callinectes* was not an appreciable cause of egg mortality except at the relatively high temperature of 20°C (ambient temp., 16°C). The fungus could cause massive mortalities (20–75% egg mortality) under certain conditions (high zoospore densities, high temperatures), but it was never observed on the eggs of *Cancer anthonyi* from nature. The *Lagenidium*-like fungus found on the crab eggs was not associated with egg mortality.

Lagenidium callinectes was originally described from the eggs of the blue crab, *Callinectes sapidus*, by Couch (1942). It has been reported from a variety of larval crustaceans (Sandoz *et al.*, 1944; Bland and Amerson, 1973; Lightner and Fontaine, 1973; Nilson *et al.*, 1975), including zoeae of *Cancer magister* (Armstrong *et al.*, 1976). The prevalence of *L. callinectes* on egg masses of *Callinectes sapidus* ranged from 0–87% of the ovigerous crabs (Rogers-Talbert, 1948). Typically, only the periphery of the egg mass was affected. Heavy infections were found in 25% of the ovigerous crabs (Rogers-Talbert, 1948). Infestation rates of *L. callinectes* were closely paralleled by those of *Carcinonemertes carcinophila* and egg mortality (usually less than 25%) fluctuated with these symbionts. Other studies have also found a relationship between the abundance of nemertean egg predators and the presence of fungal or bacterial symbionts (Wickham, 1979b; Miller and Fleming, 1983).

The physiology and nutrition of *Lagenidium callinectes* has been examined in detail (Bahnweg and Gotelli, 1980; Bahnweg and Bland, 1980). Several strains of *L. callinectes* have been isolated and their growth at different temperatures has been documented (Bahnweg and Bland, 1980). The L-1 strain of *L. callinectes* grew best at 17°C (Bahnweg and Bland, 1980), and, in the present study, it grew well at 20°C. Growth was delayed at 15°C (Rogers-Talbert, 1948). Subclinical infections were more common at lower temperatures and lower exposure levels. The 3–4 days required for the infection to spread to other eggs (Rogers-Talbert, 1948) was noted in the 20°C exposures but not at the lower temperatures. Thus, temperature had a marked effect on growth, development, and attack rates of *L. callinectes* on the eggs of *Cancer anthonyi*.

Temperature appeared to have little, if any, effect on bacterial growth and pathogenicity. Bacteria (*Leucothrix* sp. and others) contributed little to egg mortality in this study. Epibiotic bacteria are frequently found on the eggs of decapod crustaceans (Johnson *et al.*, 1971; Nilson *et al.*, 1975; Fisher and Wickham, 1976; Tharp and Bland, 1977). The role of bacteria as pathogens causing egg mortality is controversial. Bacteria have been implicated as sources of egg mortality in *Cancer magister* (Fisher, 1976; Fisher and Wickham, 1976) and *Homarus americanus* (Fisher *et al.*, 1978). Our study, controlling for the presence of other symbionts, demonstrates that bacteria are a negligible cause of egg mortality in *Cancer anthonyi*. A similar conclusion was reached for the eggs of *H.*

americanus (Harper and Talbot, 1984) and *Palaemon macrodactylus* (Fisher, 1983).

Few interactions were noted between symbionts. Nemerteans did not eat fungus-infected eggs, and they only occasionally resided on infected eggs. The worms ate eggs that were both coated and not coated with bacteria. *Lagendium callinectes* was occasionally found on the mucus, and mucous sheaths produced by *C. epialti,* and in this respect, the presence of nemerteans aided the establishment of the fungus.

Temperature plays an important role in the embryogenesis of many crustaceans (see Perkins, 1972; Wear, 1974; Amsler and George, 1984). In general, the development of *C. anthonyi* embryos was similar to that of other crustaceans. However, the embryos of *C. anthonyi* do not undergo diapause as do the embryos of *C. pagurus* (Wear, 1974). Wild (1983) found that the broods of *Cancer magister* hatched earlier at higher temperatures (17°C) than those at lower temperatures. He also showed that hatching success was inversely correlated with temperature. Curiously, hatching success was low at the ambient temperature (13°C). Mayer (1973) observed that *C. magister* embryos experienced massive mortalities at 15 and 20°C after only 3–6 days, whereas, few died at 5 and 10°C after over 11 days. *Cancer anthonyi* has a wider range of thermal tolerance because embryos survived for over 10 days at 4 and 20°C. The more southerly range of *C. anthonyi* compared to that of *C. magister* (Nations, 1975) may account for the successful development of *C. anthonyi* embryos at warmer water temperatures. Further, the lower thermal tolerance of *C. magister* embryos compared to that of *C. anthonyi* may explain why *C. anthonyi* is not abundant in northern California (Toole, 1985).

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Penetrantia clionoides, sp. nov. (Bryozoa), a Boring Bryozoan in Gastropod Shells From Guam

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Abstract. Gastropod shells from 15 sites around Guam contain specimens of a new species of boring bryozoan in the genus *Penetrantia.* This is the first boring bryozoan reported from the Mariana Islands and the first representative of the genus in the tropical Indo-West Pacific. The distinctive sculpturing on the upper surface of the operculum sets *Penetrantia clionoides* apart from other species in the genus. The new species is shown to be most similar to *Penetrantia operculata* from Hawaii. Scanning electron microscopy reveals previously unreported details of the ultrastructure of recent boring Bryozoa. Evidence of the inhibitory effects of crustose coralline algae on these borers is presented. The disputed systematic position of the boring bryozoans is discussed and the situation is shown to be complicated by the discovery here of an unusual form of aperture to the borehole, which displays both ctenostomate and cheilostomate characteristics.

Introduction

This report describes the first boring bryozoan in the genus *Penetrantia* from the tropical Indo-West Pacific. It is based on collections made around Guam as part of a larger study investigating the interactions among boring organisms (organisms that penetrate a calcareous substrate and live within it), gastropod shells, and crustose coralline algae. An unidentified borer occurred at all 15 study sites, boring mainly in the parietal region of the gastropod shells. Upon investigation, this organism was recognized as being a boring bryozoan.

The boring Bryozoa are an ethologically defined group of aberrant species that live within solid calcareous substrates in the marine environment (Pohowsky, 1978). The substrates invaded are gastropods, pelecypods, brachiopods, and crinoids. D'Orbigny (1847) was the first to describe borings in mollusc shells made by bryozoans. It was not until 1938 that the anatomy of the zooids was presented (Marcus, 1938).

There are approximately 4,000 living species of ectoproct bryozoans with as many as 15,000 fossil species (Soule and Soule, 1969a). These organisms have diversified greatly and occur in the following forms: soft gelatinous colonies, fenestrate and squamous colonies, arborescent chitinous or calcareous colonies, single- or multi-layered crustose colonies, colonies boring into hard substrates and those burrowing into soft material. The four genera that burrow through soft material are (after Pohowsky, 1978); *Hypophorella expansa* Ehlers, 1876 in polychaete tubes (*e.g., Chaetopterus* and *Terebella*); *Watersiana paessleri* Calvet, 1912 in the tunic of the colonial ascidian, *Polyzoa gordiana; Harmeriella terebrans* Borg, 1940, in the calcareous walls of cheilostome bryozoa; and *Bulbella abscondita* Braem, 1951, in rotting wood. Pohowsky (1978) reports that certain cheilostomes are known to produce shallow pits on encrusted shells (*e.g., Hippothoa divaricata* Lamoroux, 1821 and *Electra monostachys* (Busk), 1854). These latter burrowing and etching bryozoans are of interest in that they display characteristics intermediate between the true borers and the epifaunal bryozoans. Pohowsky (1978) reports that boring bryozoans penetrate exclusively by chemical means. There are, however, no data in the literature to support this statement. The four genera of Ctenostomata penetrating soft substrates are believed to employ mechanical techniques.

Because of the lack of investigation of boring bryozoans, little is known about their biology and ecology. Forty-eight species of borers (assigned to three genera) are known, but the anatomy of only sixteen has been described. The remaining species are known only by their tracings.

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Bryozoans have received little attention in the literature dealing with bioerosion. Indeed, many reviews of boring organisms fail to include any mention of the boring Bryozoa (*e.g.*, Warme, 1977). It is important to note that mollusc shells in general, and gastropod shells in particular, have been neglected in studies of bioerosion. This is the first systematic investigation of the boring organisms occurring in the three status categories of gastropod shells: living, hermited (*i.e.*, shells occupied by a hermit crab) and vacant shells. The need for such a study is obvious upon examination of the literature where, in the vast majority of publications dealing with borers in gastropod shells, the shell status is not mentioned. This leads to a loss of information regarding factors determining the longevity of a shell after death of the gastropod with consequences for conchicoles [a term proposed by Vermeij (1987) for organisms living within gastropod shells], encrusters, and for the ultimate composition of the fossil community.

In his major study of boring bryozoans, Pohowsky (1978) states that "their destructive activities appear to be far exceeded by those of boring sponges, thallophytes, and numerous other organisms." While this may be true for organisms that bore into coral, the results of this study show that it is not true for gastropods. This new species of bryozoan is the most common borer in gastropod shells on Guam, occupying 58% of all bored shells (Smyth, in prep.). In the case of bored living gastropods, 35.8% contained the bryozoan while 78.1% of bored hermited shells contained this species. In contrast, boring sponges occupied 9% of all bored shells. As this study indicates, it is clear that the importance of boring bryozoans to gastropod shells has been greatly underestimated.

Materials and Methods

During April–June 1982 and July–September 1984, living gastropods—as well as empty shells and those containing hermit crabs—were collected at 15 intertidal and shallow subtidal sites around Guam. Characteristics of the study sites will be detailed in a future paper. Areas were randomly chosen at each site and all shells within the area were collected.

After collection, a number of gastropods were maintained in the flow-through seawater system at the University of Guam Marine Laboratory for observation of the boring organisms. All shells were later preserved by oven-drying or by immersion in 4% buffered formalin or 70% ethanol. Wet-preserved shells were either immersed whole or fractured prior to preservation.

Upon return to the University of Maryland, the distribution and gross structure of the borings were determined by light microscopy. Chips were removed from whole shells using an ultra high speed precision air grinder (Dentsply Airotor, 300,000 rpm.) with Dentsply TRU-RUN (TM) carbide burs of 0.1 mm head diameter. Selected shell pieces were chosen for detailed examination of the polypide and its borehole. For anatomical studies, formalin-fixed shell chips were decalcified in a 1% solution of 12 *N* hydrochloric acid in 70% ethanol. The HCl was added dropwise to the solution as required to maintain bubbling. The shell material dissolved entirely leaving the zooids interconnected by stolons. The zooids were then teased apart in 70% ethanol containing 5% glycerol on a microscope slide, stained with methylene blue and examined using light microscopy. Line drawings were prepared from camera lucida projections. Tentacle counts were obtained by making squashes of 67 zooids. In eight cases, a clear spread was achieved, revealing the number of tentacles in the lophophore.

Chips from 21 shells were prepared for scanning electron microscopy (SEM) to examine the borehole and its associated tissues. Most of the selected chips were used directly upon removal from alcohol, though a small number of those used had been in the decalcifying mixture for various periods of time. The chips were dehydrated with a graded series of ethanols, cleaned ultrasonically and dried in a critical point dryer (Denton Vacuum, Inc., DCP-1). The chips were glued onto glass cover slips with a dilute water-soluble glue (trade name, "Elmer's Glue-All", Borden Inc.). The coverslip was then attached to a standard aluminum stub by carbon paint. The specimens were later sputter-coated under vacuum, first with a thin layer of carbon and then with gold palladium. They were viewed with a Cambridge Stereoscan Electron Microscope, Model 100. Stereo pairs were taken with a 7° tilt of the specimen. Magnifications of all SEM photomicrographs, and therefore measurements, have a ±5% margin of error.

Individual opercula were isolated by placing bored shell chips in a solution of commercial grade laundry bleach (5% NaOCl) overnight. This freed the opercula. Eleven of these opercula then underwent numerous washings in distilled water, were air-dried, manipulated with fine watchmaker's forceps, and glued onto glass coverslips. They were then prepared for SEM in the manner described above. X-ray analysis of opercula was carried out using a TN-2000 Multi-Channel Analyzer connected to a Cambridge Stereoscan Model 250 Scanning Electron Microscope operating in the Time Scan Mode. The X-rays produced were representative of the elemental composition of the sample at the point being scanned. The resulting X-ray spectrum, showing peaks indicative of concentrations of various elements, is presented as a dot distribution map.

Systematic Account

PHYLUM Bryozoa Ehrenberg, 1831
SUBPHYLUM Ectoprocta Nitsche, 1869
CLASS Gymnolaemata Allman, 1856

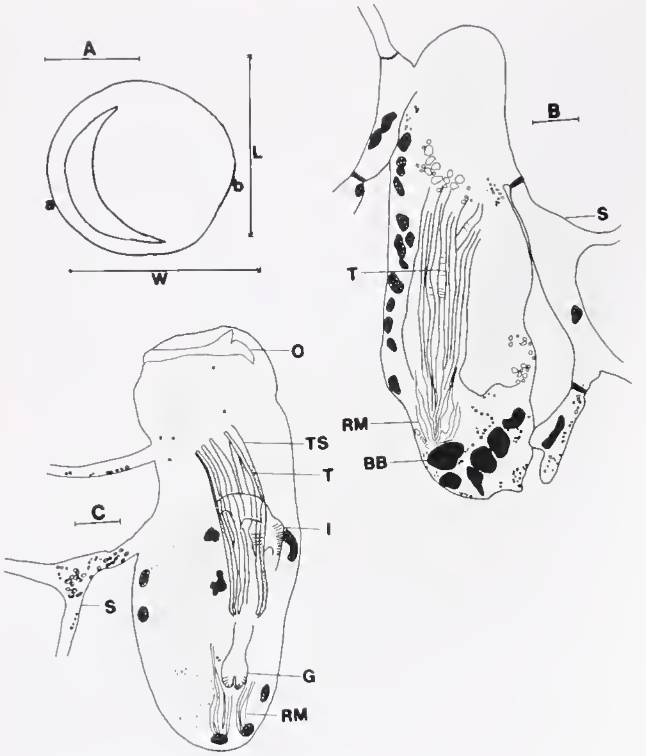


Figure 1. *Penetrantia clionoides* sp. nov. (A) Outline of operculum, indicating terminology used in text. Key: L = length of operculum, W = width of operculum. (B), (C) Anatomy of mature autozooids. Key: BB = brown body, G = gizzard, I = intestine, O = operculum, RM = retractor muscle, S = stolon, T = tentacle, TS = tentacle sheath. Scale bar = 50 μm.

ORDER Cheilostomata Busk, 1852
SUBORDER Anasca Levinsen, 1909
FAMILY Penetrantiidae Silen, 1946
GENUS *Penetrantia* Silen, 1946
Penetrantia clionoides sp. nov. (Fig. 1)

Diagnosis: Boring colonial ectoproct bryozoans with autozooids and gonozooids connected by stolons. Autozooids elongated, cylindrical; aperture closed by operculum; operculum elliptical in outline with a distinctive crescent-shaped toothed area on upper surface; tentacle number 12. Gonozooid half the length of autozooid.

Type Locality: At 0–0.5 m depth on the algal ridge and in ridge pools, Pago Bay, Guam.

Hosts: Gastropods as indicated in Appendix 1.

Type Material: Holotype (USNM 417305) and two paratypes (USNM 417306 and 417307) deposited on slides in the National Museum of Natural History, Smithsonian Institution, Washington DC.

Etymology: -oides = "resembling," derived from Gr. eidos = likeness.

Cliona-like; for the similarity of the boreholes, in surface view, to those of the boring sponge, *Cliona* sp.

Description: Each autozooid possesses a polypide bearing 12 tentacles and a typical U-shaped digestive tract. The gizzard opens into the stomach. The muscular system consists of retractor muscles, apertural muscles and parietal muscle fibers (Fig. 1B, C).

The aperture (Fig. 2A–D) is closed by an operculum, which is diagnostic. The operculum is approximately elliptical in outline with a smooth circumference and a width to length ratio of 1:1 (Fig. 1A, 3A–D).

The upper surface of the operculum consists of a shallow conical mound with a crescent-shaped area to one side of the apex of the cone (Fig. 3A). The toothed crescent (Fig. 3B), consisting of overlapping chips of calcium carbonate, lies at an angle of 30° (±5°) to the plane of the operculum. At the anterior end, these chips are grouped into larger accumulations forming teeth (Fig. 3B), the number of which varies between 14 and 16.

The undersurface of the operculum is a smooth disc with two diametrically opposed protrusions extending approximately perpendicular to the plane of the disc at its perimeter, half way between the anterior and posterior surfaces (Fig. 3D). An arced ridge connects the two protrusions and a thickened area lies on the undersurface of the lip. In one specimen, two slits were seen on the undersurface of the operculum, running parallel to and adjacent to each edge, and appearing to be scars of the opercular retractor muscles.

The average dimensions of the operculum (terminology as in Fig. 1A) are: width (W), 95.47 μm, n = 20; length (L), 90.33 μm, n = 8; depth at a, 12.53 μm, n = 4; depth at b, 4.52 μm, n = 3; maximum depth 46.35 μm, n = 3. Values for opercular length vary from 81.8 μm to

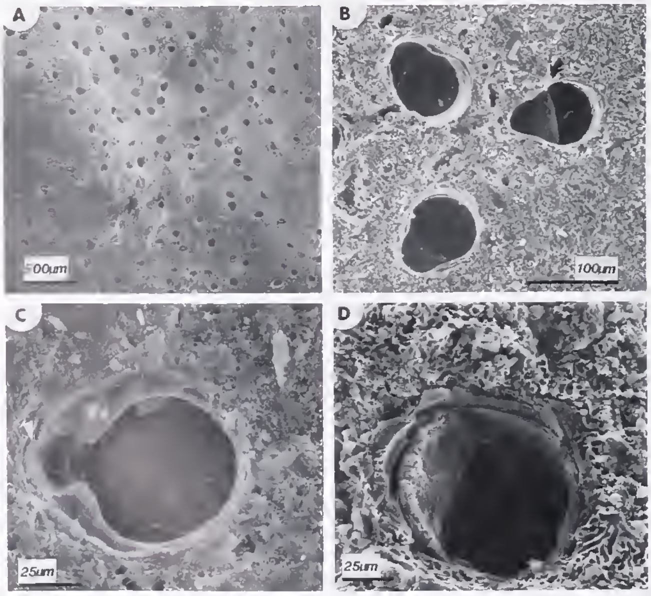


Figure 2. Borehole apertures of *Penetrantia clionoides* sp. nov. (A) Shell surface showing characteristic shape of borehole apertures. (B) Typical borehole apertures with one operculum open. (C) Rare keyhole-shaped borehole aperture showing ascophoran cheilostome-like anter and narrower poster (arrow). (D) Open operculum.

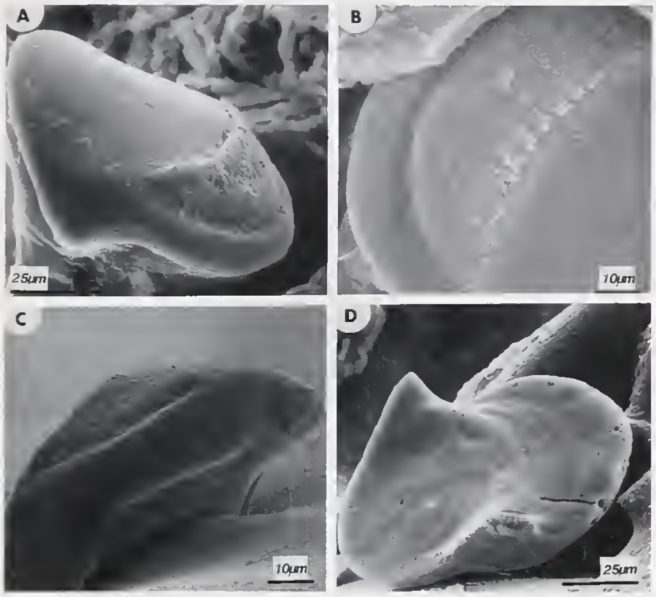


Figure 3. Loosened opercula of *Penetrantia clionoides* sp. nov. (A) Dorsal view of operculum. (B) Detail of toothed ridge on anterior surface of operculum. (C) Lip on anterior end of operculum. (D) Ventral view of operculum showing the two diametrically opposed protrusions.

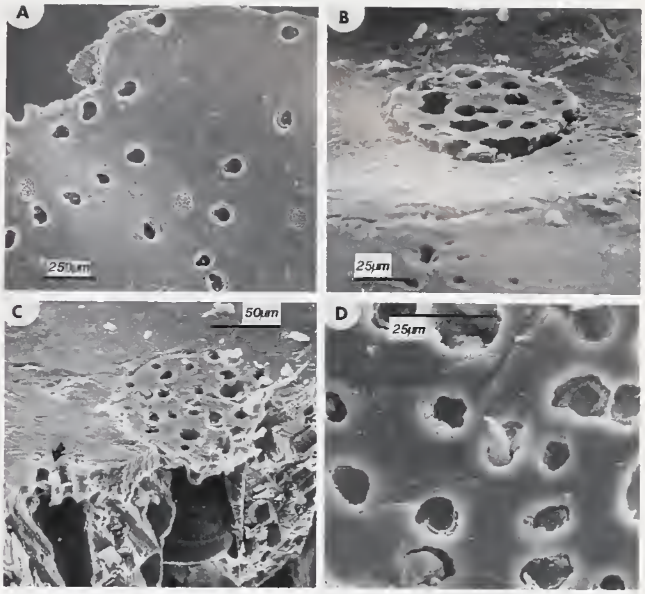


Figure 5. Borehole apertures and sieves of *Penetrantia clionoides* sp. nov. (A) Surface view of borehole apertures, sieves and individual holes. (B) Sieve. (C) Sieve over fully formed polypide complete with operculum. Note early stage of bryozoan borehole (arrow). (D) Detail of sieve in 'C'.

97.1 µm and for width vary from 68.8 µm to 108.9 µm. The operculum is thinnest at its hind edge and thickest at the rear of the crescent.

Examination of the dot distribution maps indicates that the opercula are composed mainly of calcium carbonate with traces of sodium, zinc, magnesium, and aluminum.

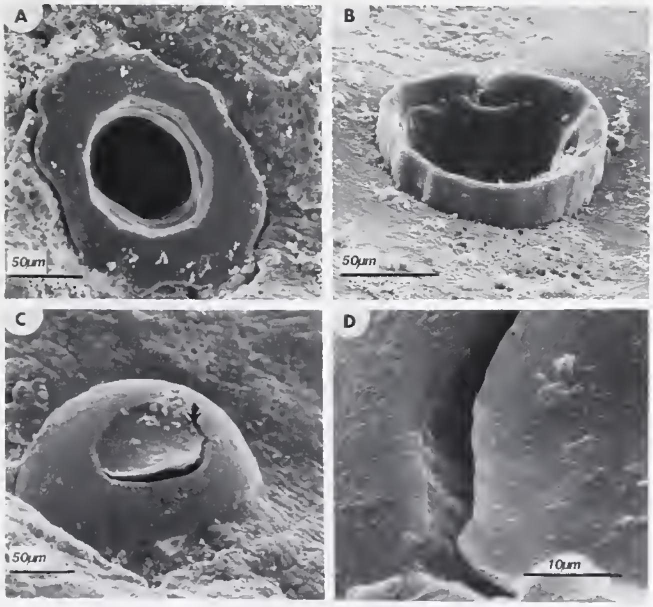


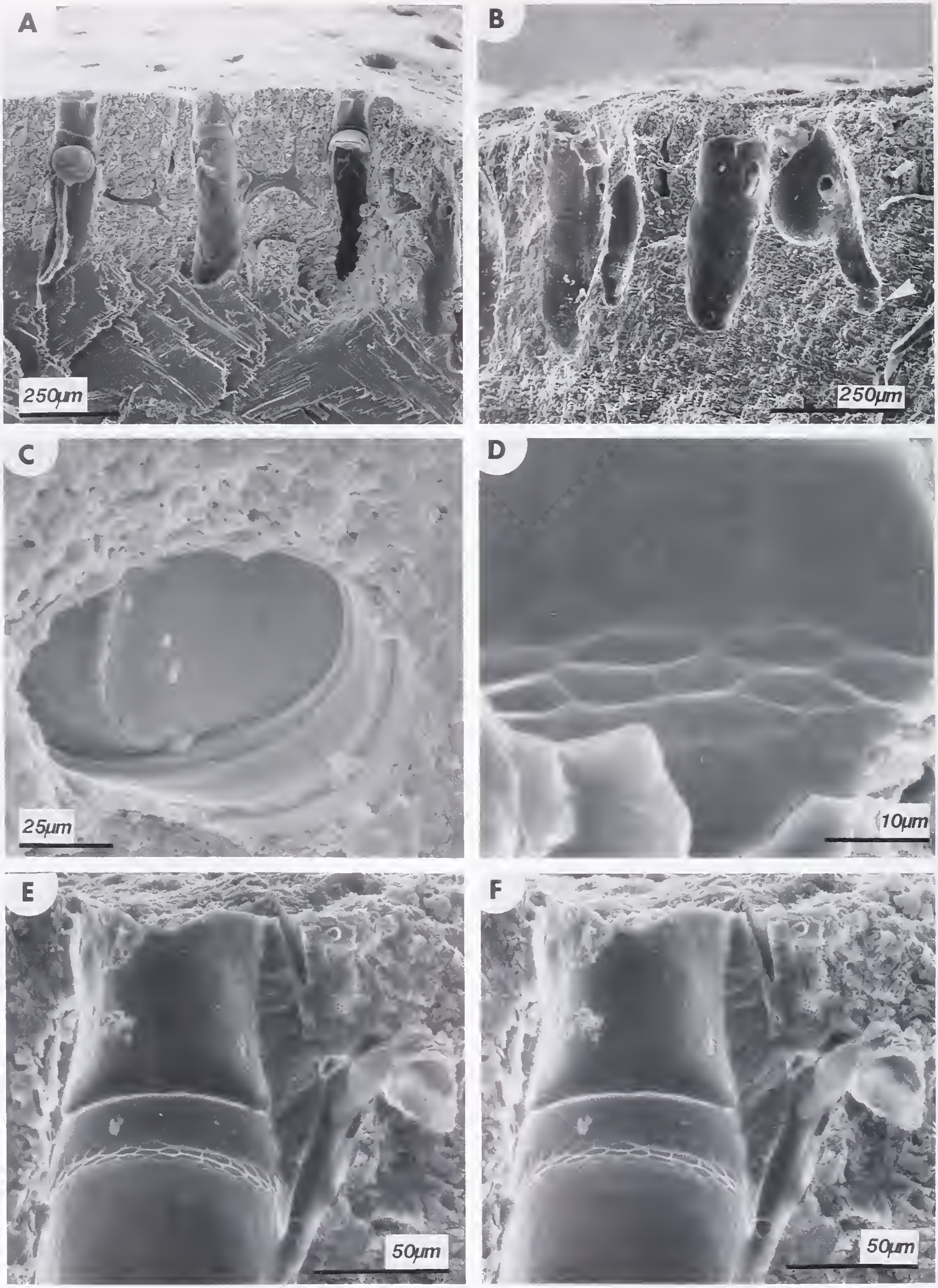
Figure 4. Unusual borehole apertures of *Penetrantia clionoides* sp. nov. (A) Calcareous irregular margin around aperture. (B) Raised margin. (C) Plug in calcareous margin. (D) Higher magnification of 'C' (arrow).

Burrow apertures occur randomly on the outer surface of the mollusc shell (Fig. 2A). Statistical analyses (G-test of goodness of fit) show that even in colonies in the early stages of development, the pattern is random.

The shape of the aperture of the borehole is variable, from approximately circular to keyhole-shaped (Fig. 2A–D). In the latter case, the crescent lies in the narrow region of the aperture (Fig. 2B). These apertures are of the typical ascophoran cheilostome form with an anter and poster (Fig. 2C).

On rare occasions, the lining of a borehole is raised above the surrounding substrate (Fig. 4B). In only eight cases, an irregularly shaped calcareous margin was seen around a borehole aperture (Fig. 4A). Three of these margins are plugged in the center (Fig. 4C, 4D), not with an operculum but with a structure that appears to be about to detach (Fig. 4C).

Shells bored by *P. clionoides* are often seen with a number of white, circular areas (henceforth called "sieves") on the shell surface. These are perforated and of equivalent size to that of the borehole apertures (Fig. 5A–D). Interspersed among these sieves are accumulations of small holes, each hole equivalent in size to the individual holes making up a sieve (Fig. 5A). By fracturing one of the sieves, perpendicular to the shell surface, it was revealed that the sieve lies above a fully formed polypide complete with operculum (Fig. 5C). A long, narrow borehole next to this may represent the early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stage of a bryozoan borehole (Fig. 5C, arrow). The data indicate that sieves occur where more than two stolons meet.

The borehole of the autozooid lies perpendicular to the shell surface and is cylindrical with a bluntly rounded proximal end (Fig. 6A, B). The average dimensions are: depth of borehole, 410.24 μm, n = 36; width of borehole, 103.49 μm, n = 25.

The inner surface of the borehole is featureless except for a narrow zone beginning, on average, $\frac{1}{6}$ of the way along its length from the distal end. At its most distal end this zone consists of a thin ledge lying 72.2 μm, n = 6, below the shell surface (Fig. 6E, F). This ledge is, on average, 28.2 μm thick, n = 6. The upper surface of the operculum abuts against the undersurface of this ledge (Fig. 6C). The proximal end of this zone has a mesh-like appearance (Fig. 6D). This meshed area sometimes extends down entirely from the ledge. A nodule protrudes as part of the ledge at the proximal end of this meshed area and functions in articulation with the operculum (Fig. 7B). The operculum is closed by a pair of occlusor muscles, one arising on each side of the zooid and converging to a tendon which inserts on the operculum. The occlusor muscle inserts behind the protrusions (Fig. 7B).

The operculum is attached to the zooecium along its entire perimeter. At the posterior surface, a muscular connection links the operculum to the zooecium (Fig. 7A, 7F). This tissue is exposed when the operculum is open.

When the operculum is open, its posterior end protrudes from the borehole with the anterior end positioned out of view inside the borehole (Fig. 2D). As a result of examination of opercula found in the open position it appears that the operculum pivots on its two protrusions. These appear to articulate with the nodules seen on the wall of the borehole (Fig. 7B–E).

Occasionally, boreholes occur in such proximity to each other that only a thin area of shell remains between them. Up to 60% of the boreholes in a shell may be devoid of the polypide.

On average, the borehole of the gonozooid (Fig. 8B) is half the depth of the autozooid. Gonozooids include a bulbous embryo chamber which appears to emanate on either the right or the left side of the gonozooid, though it is possible that some of the samples observed are complementary fracture faces.

Boreholes differing from the usual cylindrical type are occasionally seen. These have an additional chamber on the proximal end that may represent a growing edge. There are also shorter and wider boreholes, in the shape of gonozooids, but with the extension on the proximal end. These appear to be heterozooids (Fig. 8A).

Stolons emanate from zooids by a lateral branch at the distal end. Stolonal tracings are not seen on the shell surface until after soaking in acidic solution (Fig. 8C, 8D). In the intact shell, then, the only indication of the presence of *P. clionoides* is the occurrence of borehole apertures on the shell surface. On average, the diameter of a stolon is 9.8 μm, n = 6.

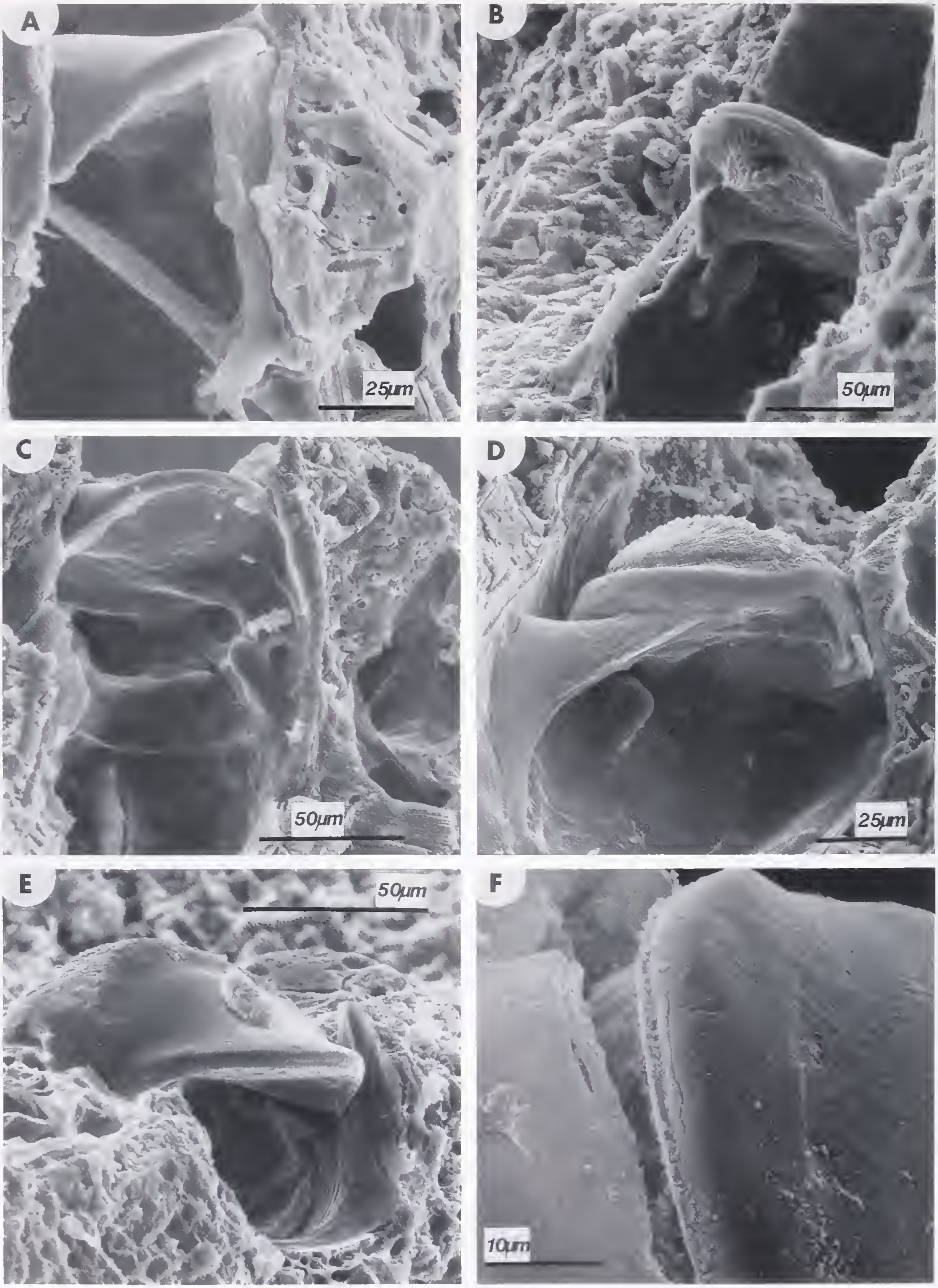
Penetrantia clionoides may occur in association with one or more of the other boring organisms (acrothoracican barnacles, clionid sponges, polydorid polychaetes, and the boring foraminiferan, *Cymbaloporella tabellaeformis*) occurring in gastropod shells at these study sites (Smyth, in prep.).

The susceptibility of *P. clionoides* to suffocation by overgrowth is indicated by its inability to maintain openings through coralline algae. A case of confinement, with corallines overgrowing a borehole aperture and trapping the bryozoan, is seen in Figure 9A. The other boring organisms in this study are capable of maintaining openings through corallines. Detailed examination of the parietal wall area of the shell (area on right side of shell aperture) has resulted in the discovery of a number of bryozoan boreholes enveloped by recently deposited shell material. As the shell grows, the boreholes become absorbed into the parietal wall area (Fig. 9B). In a living specimen of *Drupa morum* a bryozoan aperture, on the upper right side of the shell aperture, was in the process of being overgrown by the smooth shell of the outer lip (Fig. 9B). Clearly, this is a transient stage, but enveloped boreholes are often seen along the margin of the parietal wall and the inner lip (that is, where the smooth shell of the parietal wall meets the rough, encrusted shell of the inner lip area). Using light microscopy, these borehole apertures can be seen as white, translucent spots in the shell material.

Observations of broken opercula indicate that the operculum is hollow (Fig. 9C). This hollow region is lined with crystals resembling those of the crescent in structure. A fracture in the protrusion on the side of an operculum shows that these flanges are not hollow.

A number of bryozoan boreholes, devoid of polypide and operculum, were occupied by unusual crustacean-like organisms as yet unidentified (Fig. 9D). These bear some resemblance to harpacticoid copepods (pers. comm.; R. Cressey, Curator of Crustacea, Smithsonian Institution).

Figure 6. Boreholes of *Penetrantia clionoides* sp. nov. (A) Three boreholes in section. (B) Detail of 'A' showing ledges in borehole and stolonal tracings. (C) Upper surface of operculum abutting against undersurface of upper ledge. (D) Meshed area at top of borehole. (E), (F) Stereo pair of meshed area at top of borehole taken at 7° tilt.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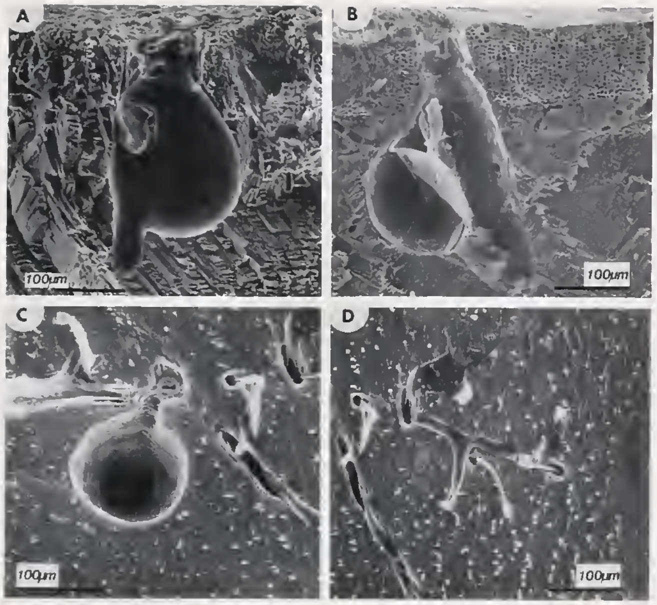

Figure 8. Stolons and unusual boreholes of *Penetrantia clionoides* sp. nov. (A) Probable heterozooid borehole. (B) Gonozooid with bulbous embryo chamber on left and operculum. (C) Stolons and section through boreholes in acid-treated shell. (D) Stolons on surface of acid-treated shell.

As a hermited shell is carried by its occupant, those corallines on the left of the aperture become worn off as a consequence of abrasion with the substrate. This results in a bare patch extending ventrally along the periphery of the aperture, which is invaded by the bryozoan.

Remarks

Penetrantia clionoides can be distinguished from all other members of the genus by its distinctive operculum. The species to which *P. clionoides* is most similar is *P. operculata* described from Haena Bay, Kauai, Hawaii (Soule and Soule, 1969b). The operculum of *P. operculata* is described as "a massive knobbed operculum" though no dimensions are presented. This operculum differs from that of *P. clionoides* in the absence of the crescent and of the other distinctive features of the operculum of *P. clionoides*. The differences between the latter operculum and that of *P. operculata* are major. Pouyet and David (1979), working with the genus *Steginoporella*, point out that opercular differences provide justification for separation into distinct species.

Discussion

Presently, it is generally accepted that the genus *Penetrantia* belongs in the suborder Anasca of the Cheilostomata. An operculum is characteristic of cheilostome bryozoans (Ryland, 1970). Nevertheless, scant attention appears to have been paid to its shape, composition, and method of articulation. This report presents the first detailed study of a recent bryozoan operculum including the first SEM micrographs of such an operculum. The operculum of *P. clionoides* is shown to be calcareous. Totally or partially calcareous opercula are apparently very rare in recent Cheilostomata (Voigt, 1974). There appear to be a number of similarities with the operculum of *Inversaria tubiporacea* from the Dutch Maastrichtian Chalk-tuff. Voigt (1974) points out that this operculum deviates in its shape to such a large extent from the opercula of recent Cheilostomata that, in isolation, it would never be taken for a bryozoan operculum but for a problematic microfossil. The conical outgrowth on the upper surface of the operculum of *P. clionoides* and the protrusions on the undersurface are, to a degree, comparable to those on *I. tubiporacea*.

There is no agreement as to the systematic position of boring bryozoans. Soule and Soule (1969a) removed the Penetrantiidae to the Cheilostomata but the most recent major study of the group (Pohowsky, 1978) regards all boring bryozoans as members of the order Ctenostomata, though with slight reservations regarding the Penetrantiidae. Representatives of the order Cheilostomata possess one or more of the following features: an ovicell, operculum, and calcified body wall. Pohowsky (1978) concedes that all three of the cheilostomatous characters are present in *Penetrantia densa* Silen, but argues that this is an example of convergence with the cheilostomes. Voigt (in Voigt and Soule, 1973) believes that a ctenostomatous relationship for *Penetrantia* cannot be totally excluded. In summary, the literature suggests that either the boring Bryozoa are a small obscure group specialized for the boring mode of existence (as in Pohowsky, 1978) or that boring constitutes an important ecological niche for bryozoans (as in Soule and Soule, 1969a). It is clear that this taxonomic issue cannot be resolved until the anatomy of the various borers is investigated in detail. For the moment, I accept the classification presented by Soule and Soule (1969a), though with some reservations because it is clear that characters have arisen independently in more than one lineage of bryozoans.

Figure 7. Articulation of opercula of *Penetrantia clionoides* sp. nov. (A) Operculum *in situ* showing opening and closing mechanism. (B) Posterior view of operculum showing tendon inserting on operculum and articulation with nodule. (C) Posterior view of operculum. (D) Operculum showing nodule and part of zooecial attachment. (E) Operculum in open position with posterior end protruding. (F) Attachment of operculum to zooecium at perimeter.

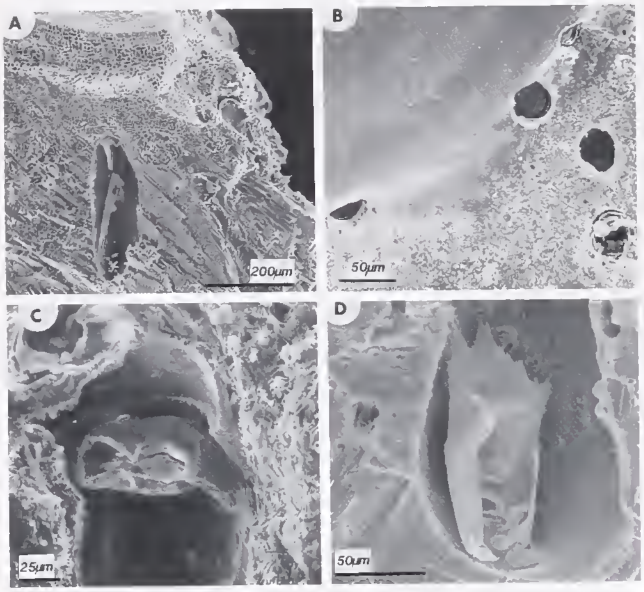

Figure 9. Overgrowth of *Penetrantia clionoides* sp. nov. (A) Borehole overgrown by crustose coralline algae. (B) Boreholes enveloped by recently deposited shell material in parietal wall area of shell. (C) Broken operculum indicating its hollow nature. (D) Empty borehole occupied by unidentified crustacean nestler.

Further investigation may show that because *P. clionoides* has features that overlap so much between ctenostomate and cheilostomate characters, a major reclassification is necessary. It seems that the generally accepted classification must eventually be abandoned. This study has introduced some data that make the issue of the systematic position of these boring bryozoans more confused. The presence of the rare keyhole-shaped burrow apertures is such a factor. These apertures are of the typical ascophoran cheilostome form with an anter and poster in the operculum. In the ascophoran aperture, the anterior part (anter) covers the passage for the polypide and the posterior part (poster) simultaneously covers the inlet to the compensatrix (compensation sac). Water enters the compensation sac to equalize space occupied by the polypide. The presence or absence of a compensation sac has been important for determining the appropriate suborder (Anasca, Ascophora). *Penetrantia clionoides* has some characteristics of both orders and suggests the inappropriateness of the criteria on which these orders are based. This problem has been addressed in the past. In fact, Silen (1942) combined the ctenostomes with the cheilostomes as the Cheiloctenostomata. Ryland (1970) suggested that the division into the two suborders, Anasca and Ascophora, is invalid. He recognized four major divisions in the Cheilostomata. As Bassler (1953) indicated, "a classification of the Bryozoa that finally may be judged acceptable is impossible at present, because probably each family should be characterized essentially by the larval form of its constituents; unfortunately, this is known at present for comparatively few families."

The opercula are remarkably non-encrusted relative to other structures I have examined on the surface of these shells. The movement of the operculum itself or some behavioral or chemical feature of the organism may be responsible for this.

The function of the plug in the calcareous margins around the burrow apertures has not yet been determined. I suggest that such a structure might be a closure device sealing off the polypide as it undergoes regeneration. However, the low incidence of these is puzzling in light of the purported high frequency of regeneration in boring bryozoans.

Budding pattern is defined by Pohowsky (1978) as "the geometric and sequential relationship existing among the stolons and zooids in a colony." It is generally assumed that boring bryozoans have a characteristic non-random budding pattern that is distinctive and easily recognized on the shell surface. It became evident during this study that such an assumption can be misleading. I witnessed situations where the presence of boring bryozoans is discounted because the budding pattern displayed by the borer does not conform with that previously published for bryozoans.

As a consequence of their appearance and the absence of the expected non-random "characteristic" budding pattern, the boreholes were initially assumed to be young clionid borings. However, fracturing the boreholes revealed the presence of opercula plugging the borehole apertures. This eventually lead to the determination of the bryozoan nature of the burrows. Boekschoten (1966) reported a similar confusion in surface view.

The boring habit is generally assumed to have arisen as a means of refuge; organisms that bore are less vulnerable to predation. While *P. clionoides* is protected by being recessed in the shell material and by the presence of the operculum, there remains a degree of vulnerability. The main sources of mortality for these organisms are overgrowth/suffocation and predation. In this respect, it is interesting to note that the zooids of boring bryozoans are much less closely packed than are zooids of other bryozoans. The adaptive value of this is that if encrustation and overgrowth occur, there is a greater likelihood that part of the colony will survive to reproduce. Of the variety of boring organisms occurring in gastropods at these study sites, only the boring Bryozoa appear to be susceptible to interference by the gastropod. The tendency of these organisms to bore in the inner lip area leaves them in a transient situation where, unless the gastropod is dead or has reached its terminal growth stage, they will eventually be absorbed into the shell material as the gastropod grows. Hermited shells provide a more stable environment in terms of lack of interference from the shell-dweller. However, other factors come into play—such as the greater likelihood of being overgrown by encrusters—in hermited shells. Their susceptibility to suffocation by overgrowth is indicated by the discovery of their inability to maintain openings through coralline algae. Thus it is clear that a calcium carbonate substrate is not always a source of protection for organisms living within it. Abrasion, borings, fracture, and encrustation are among the problems encountered.

There is no evidence of the existence of predators on Guam that gain access by drilling through the operculum. Having observed thousands of opercula, not one drillhole was seen. Taylor (1982) records pycnogonids and nudibranchs as major predators of Bryozoa, but no evidence of the effects of these specialized predators was seen in this large collection. Cairns (1982) saw circular boreholes in a calcareous stylasterine operculum from the Antarctic, but he argues against a molluscan predator without offering any alternative suggestions. The fact that the operculum is recessed may reduce the likelihood of predation by drilling. Presumably, many predators are unable to cope with the operculum. Hayward and Ryland (1969) report that some dorids push the operculum aside or into the zooid, and Ryland (1976) shows that it may be torn out. Pycnogonid predators allow a polypide to emerge and then rapidly dart the proboscis through the orifice and devour the contents (Wyer and King, 1973). Apparently, this has been observed only for the suborder Anasca. The zooids are attacked singly and are presumed to regenerate polypides later.

Vermeij (1978) documented a Mesozoic revolution in marine faunae which he attributed largely to the evolution of new predators. Larwood and Taylor (1981) show that evidence from the Bryozoa provides support for Vermeij's hypothesis because several features of Mesozoic bryozoan evolution can be interpreted as responses to increasing levels of predation. An example is the evolution of operculate autozooids and mandibulate polymorphs in two independent groups (cheilostomes and melicerititid cyclostomes). These authors point out that predation pressure may have been an important selective force operating on bryozoans during the Mesozoic, although direct evidence of paleopredation is limited.

There is no evidence that boring bryozoans are parasitic on mollusc tissue (Pohowsky, 1978). Pohowsky further shows that their occurrence in empty shells indicates their non-parasitic nature. In the case of the gastropod shells in this study it is clear that, as a consequence of the dimensions of the borings, they do not penetrate sufficiently deep to come into contact with gastropod tissue.

As a consequence of the small size of these zooids, the shell weakening effects would not be expected to be great. However, the tendency to concentrate in high density on the left side of the aperture must make this area more vulnerable to peeling or crushing predators.

Vacant bryozoan boreholes provide a space resource for other organisms in the habitat. The existence of these nestlers emphasizes the complexity of issues associated with boring: not only the issues of bioerosion and consequent effects upon the bored substrate but also the additional space resource provided for other organisms.

The concentration of bryozoan boreholes in areas on hermited shells where the coralline algae are worn away is of great significance. This negative association between boring bryozoans and crustose coralline algae was discovered during an investigation of interference, by corallines, with the settlement of larvae of boring organisms on gastropod shells (Smyth, in prep.). Most shells in this collection come from areas with high surf, where crustose coralline cover is intense. Hermited shells are more heavily encrusted with corallines than are living gastropods, in part because of their greater age. If boring Bryozoa are seen on a hermited shell, they are seen only in the bare areas on the left side of the aperture. Examination of surrounding areas of the shell, under the coralline, rarely reveals evidence of the Bryozoa having been overgrown by the corallines. When bryozoan borings are seen in living gastropods it is, again, only in bare patches. In the latter case, the patches are scattered over the shell surface.

Future work includes examination of the larvae and observations of their settlement behavior, field experiments to determine the magnitude of shell weakening and vulnerability to peeling or crushing predators, and further studies to clarify the systematic position of the group.

Acknowledgments

Thanks to Dr. J. D. Soule for assistance with identification of structures seen in scanning electron micrographs during the preliminary stages of this study. Field work was supported by a Smithsonian Predoctoral Fellowship and by the Lerner Gray Fund. Thanks to Drs. Steve Cairns, Alan Cheetham, Geerat Vermeij, and Judith Winston for discussions during the project.

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Appendix 1

Species of gastropod collected at 15 study sites around Guam

Gastropod	Presence/Absence of *Penetrantia clionoides*
Bursa bufonia (Gmelin, 1791)	+
Bursa cruentata (Sowerby, 1835)	+
Bursa sp.	+
Bursa verrucosa (Sowerby, 1825)	+
Cantharus sp.	+
Cantharus undosus (Linnaeus, 1758)	+
Casmaria ponderosa (Gmelin, 1791)	+
Cellana radiata orientalis (Pilsbry, 1891)	–
Cerithium nodulosum Bruguière, 1792	+
Cerithium sp.	+
Chicoreus brunneus (Link, 1807)	+
Chicoreus sp.	+
Conus sp.	+
Coralliophila violacea (Kiener, 1836A)	+
Cowrie	+
Cymatium nicobaricum (Roeding, 1798)	+
Drupa arachnoides Lamarck, 1816	+
Drupa clathrata Lamarck, 1816	+
Drupa morum Roeding, 1798	+
Drupa ricina (Linnaeus, 1758)	+
Drupa rubisidaeus Roeding, 1798	+
Drupa sp.	+
Drupella elata (Blainville, 1832)	–
Lambis sp.	–
Latirus barclayii (Reeve, 1847)	+
Morula granulata (Duclos, 1832)	+
Morula sp.	+
Morula uva (Roeding, 1798)	+
Nassa serta (Bruguière, 1789)	+
Patella flexuosa Quoy & Gaimard, 1834	–
Sabia conica (Schumacher, 1817)	+
Strigatella paupercula (Linnaeus, 1758)	+
Strombus luhuanus Linnaeus, 1758	–
Strombus mutabilis Swainson, 1821	–
Strombus sp.	+
Tectus pyramis (Born, 1778)	+
Trochus maculatus Linnaeus, 1758	+
Trochus niloticus Linnaeus, 1767	+
Trochus ochreleucus Gmelin, 1791	+
Trochus sp.	+
Thais armigera Link, 1807	+
Thais intermedia Kiener, 1836	+
Thais sp.	+
Thais tuberosa Roeding, 1798	+
Turbo setosus Gmelin, 1791	+
Vasum ceramicum (Linnaeus, 1758)	+
Vasum sp.	+
Vasum turbinellus (Linnaeus, 1758)	+

Presence or absence of *Penetrantia clionoides* in that particular gastropod species indicated by "+" or "–", respectively. Rare species (sample size < 10 shells) excluded.

Reference: *Biol. Bull.* **174:** 287–302. (June, 1988)

Reproductive Patterns in Six Species of *Lepidochitona* (Mollusca: Polyplacophora) from the Pacific Coast of North America

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Abstract. Reproductive patterns are documented and compared in six morphologically similar members of the chiton genus *Lepidochitona* from the west coast of North America (Oregonian and Californian Provinces). Three of the six species studied brood embryos: *L. thomasi* (Pilsbry, 1898), *L. caverna* Eernisse, 1986, and *L. fernaldi* Eernisse, 1986. The offspring of brooders are able to crawl away. In contrast, *L. dentiens* (Gould, 1846), *L. hartwegii* (Carpenter, 1855), and the less common *L. berryana* Eernisse, 1986 are free spawners whose offspring are obligate dispersers. The dispersal consequences of brooding or not brooding are exemplified by *Lepidochitona,* without major complications due to differences in larval size or larval feeding ability. Developmentally, brooders and free spawners in *Lepidochitona* differ primarily in stage (*i.e.,* age) at which larvae hatch from their egg capsules. Larval size and morphology differences are present but not as extreme as in other taxa.

As in many other taxa there is a link between brooding and particular life history traits, especially small adult size and self-fertilization. Size comparisons match the expectation that, as adults, brooders are generally as small or smaller than free spawners. The two smallest of the three brooders, *L. caverna* and *L. fernaldi,* are also simultaneous hermaphrodites, based on examination of gonads. These are the only known hermaphroditic chiton species, and are apparently fully capable of self-fertilizing multiple broods based on evidence from animals isolated for up to nine months in the laboratory. The third brooder, *L. thomasi,* is more typical of chiton species including those that brood; it has separate sexes and does not produce viable broods in isolation. Based on comparisons among chitons and among other groups that normally have separate sexes, hermaphroditism is argued to be a consequence of brooding, rather than the reverse. A mechanism is suggested that would link hermaphroditism, but not small adult size, to the consequences of crawl-away offspring. Small adult size could alternatively be attributed to the morphological constraints imposed by brooding.

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Introduction

A marine embryo, free spawned by a benthic animal into the plankton, is unlikely to return and settle where it was initially released. In contrast, some benthic marine animals brood embryos, often protecting them until they emerge as crawl-away offspring. Brooders, or egg-capsule layers, that have crawl-away offspring (called "brooders" hereafter) often share particular life history traits relative to free spawners, or even relative to animals that brood but then release larvae before they are competent to settle (*e.g.,* barnacles). The traits associated with brooders typically include small adult size and large egg size, corresponding to the release of relatively few large offspring that depend on yolk, rather than on planktonic feeding (Menge, 1975). Brooding has also been linked to hermaphroditism. At least in taxa whose majority of species are gonochoric (*i.e.,* have separate sexes), the exceptional hermaphroditic species invariably have crawl-away offspring and also tend to self-fertilize regularly (Strathmann *et al.,* 1984), unlike the vast majority of marine hermaphroditic animals (*e.g.,* barnacles, nudibranchs, tunicates) that have effective blocks to self-fertilization.

Defining the contrast between brooders and free spawners in terms of the likelihood an offspring will remain near its parent emphasizes the hypothesized differences such an opportunity might present. It would be incorrect, however, to attribute all life history traits shared by brooders to their unique opportunity to crawl away. It is important to also recognize that certain traits may be influenced by the morphological and behavioral constraints of brooding itself. Moreover, differences between free spawning and brooding species can alternatively be explained by selection for planktonic feeding ability at the larval stage, or as a result of historical or ecological constraints on morphology. Yet there may be particular traits correlated to brooding that can be attributed specifically to the expected consequences of permitting one's offspring to remain nearby.

Brooding is distributed among ten quite distinct chiton genera (Pearse, 1979; this study). Given this presumed parallel evolution of brooding, comparisons among chitons might reveal shared patterns of brooding with other life history traits. Brooding is especially common in the genus *Lepidochitona.* No fewer than 6 of the 20 or so members of this genus are known to brood, including the subject of Kowalevsky's (1883) well-known embryological study, the Mediteranean species *Chiton polii* Philippi [=*L. corrugata* (Reeve)]. Also long known are two brooders studied by Heath (1899) as *Nuttallina thomasi* Pilsbry [=*L. thomasi* (Pilsbry)] and *Trachydermon raymondi* Pilsbry [=*L. caverna* Eernisse]. *L. caverna* is also noteworthy as the first reported hermaphroditic chiton species (Heath, 1907). Another brooder, *L. fernaldi* Eernisse, 1986, is distinguished herein as a second case of hermaphroditism in chitons. Adding to the list of brooders, Kaas and Strack (1986) have recently reported brooding in the West African species, *L. caboverdensis* Kaas and Strack, and H. L. Strack (in litt., 1986) has discovered brooding in the Canary Islands endemic species, *L. stroemfelti* (Bergenhayn).

In this study I document reproductive patterns in six morphologically similar species of *Lepidochitona* from populations along the west coast of the United States, including three of the brooders mentioned above: *L. thomasi, L. caverna,* and *L. fernaldi.* For all six species I emphasize: (*i*) evidence for free spawning or brooding; (*ii*) comparisons of early development; (*iii*) likelihood that offspring can crawl away; (*iv*) intra- and interspecific comparisons of size; and (*v*) evidence for gonochorism (*i.e.,* separate sexes) or hermaphroditism; and (*vi*) the possibility that individuals might produce viable embryos (*i.e.,* by self-fertilization or parthenogenesis) when isolated from other individuals. Using comparisons among *Lepidochitona,* I then explore hypotheses linking particular life history traits to brooding. These hypotheses may be especially relevant to patterns observed in other, normally gonochoric, marine invertebrate taxa.

Materials and Methods

Between January, 1980, and October, 1985, chitons were collected from a variety of locations in California and Washington (Figs. 1, 2, and Table I). Most animals were kept alive for observation at Long Marine Laboratory, Santa Cruz, California, or at Friday Harbor Laboratories, Friday Harbor, Washington. Animals transported from the field were supported by a small air pump and bubbler, cool temperatures, and frequent water changes. Aeration was especially helpful because chitons crawl out of oxygen-depleted water.

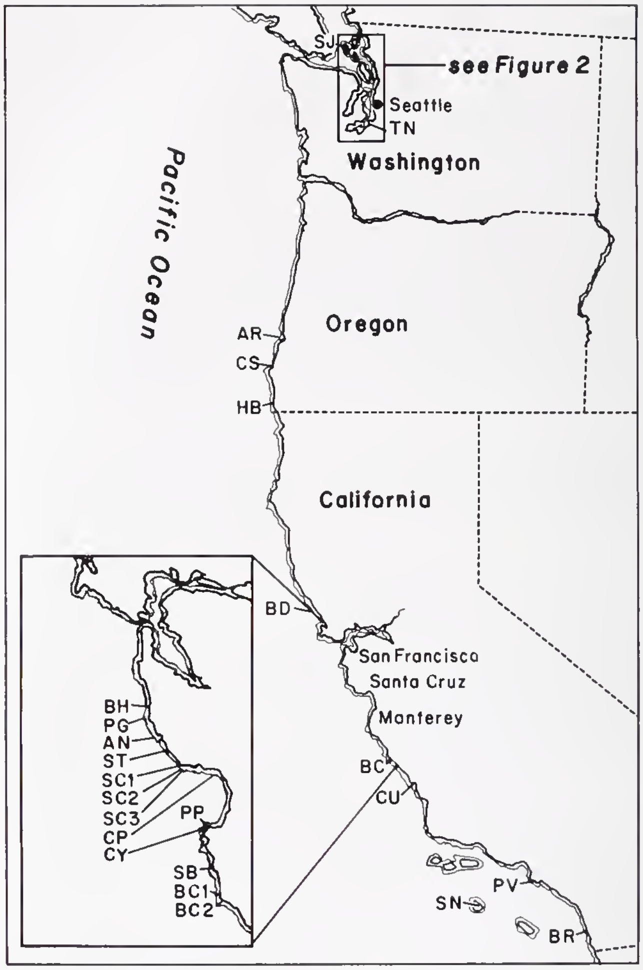

Figure 1. Primary study sites, abbreviated as follows (listed approximately north to south): In San Juan Co. (Washington): SJ = San Juan Is., west side (see Fig. 2). In Pierce Co.: TN = Tacoma Narrows. In Sonoma Co. (California): BD = Bodega Head. In San Mateo Co.: BH = Bean Hollow (Arrojo de los Frijoles); PG = Pigeon Pt.; AN = Año Nuevo Pt. In Santa Cruz Co.: ST = Scott Creek; SC = West Cliff Drive in Santa Cruz including SC1 = Auburn Ave.; SC2 = Stockton Ave.; SC3 = Pt. Santa Cruz; CP = Soquel Pt., Capitola. In Monterey Co.: PP = Pt. Pinos; CY = Cypress Pt.; SB = Soberanes Pt.; BC = Landels-Hill Big Creek Reserve. In Ventura Co.: SN = San Nicolas Is. In Los Angeles Co.: PV = Palos Verdes. In San Diego Co.: BR = Bird Rock, La Jolla.

Over-zealous collecting was avoided, especially at localities where chitons occupied tightly packed barnacle hummocks, because destructive sampling was then required to obtain chitons. The pointed end of a Diamond-Deb™ fingernail file was an excellent collecting tool, su-

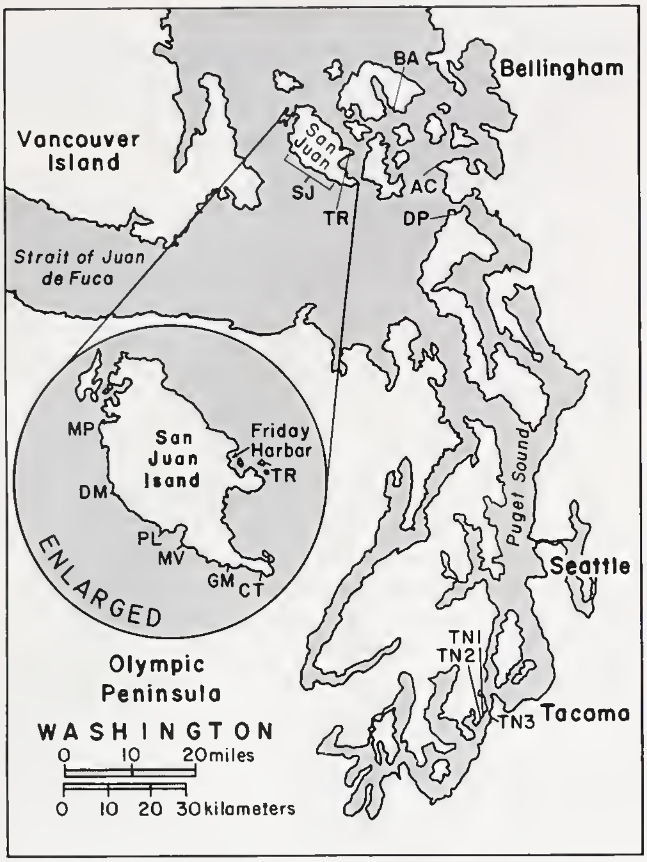

Figure 2. Study sites in San Juan Islands and Puget Sound, abbreviated as follows: SJ = San Juan Is., west side, including MP = Mitchell Pt.; DM = Deadman Bay; PL = Pile Pt.; MV = Mar Vista Resort; GM = Grandmother's Cove; CT = Cattle Pt. Also in San Juan Co. are: TR = Turn Rock; BA = Barnacle Rock. In Skagit Co.: AC = Shannon Pt., Anacortes. In Island Co.: DP = Deception Pass. In Pierce Co.: TN = Tacoma Narrows, including TN1, 2 near bridge on west side and TN3 at Salmon Beach, east side.

perior to conventional collecting blades or spatulas for small chitons. For a record of the percentage of individuals brooding (Fig. 3), all specimens of each species taken were routinely examined for brooded embryos in the pallial groove on each side of the foot. Chiton length from the most anterior to the most posterior margin of the girdle was measured in the lab by first allowing the animal to extend itself naturally on a flat surface. Length is an adequate descriptor of size for relative comparisons within and between species, but is somewhat complicated by the characteristic width and profile of each species and a shape change; individuals generally become wider and higher in profile with increasing size (Eernisse, 1984).

Size frequencies and descriptive statistics were calculated for each species from one or more population(s) (Figs. 4, 5). *L. caverna, L. fernaldi,* and *L. thomasi* were often brooding when collected, so the size of the brooding individuals is presented relative to the size frequency of all individuals measured (Figs. 5b, d, f). The collection of individuals for measurement is undoubtedly biased towards mature-sized specimens, because juveniles are inconspicuous and adults were taken preferentially.

Free-spawning species were kept in the splash tanks or, for short periods, in closed plastic containers filled nearly full with filtered seawater. This water was checked daily for evidence of spawned gametes and replaced daily with clean seawater. Observations were most detailed for *L. dentiens;* 220 adults were observed in the lab on various occasions for at least a few days after collection.

Additional specimens of *L. dentiens,* collected on six occasions from three locations in central California (BH, SC, and Mission Pt., Carmel), were prepared for histological analysis. Oocyte size frequencies were calculated for 16 females. For histological analyses of *L. dentiens* and other species, animals were relaxed in 7.3 percent aqueous $MgCl_2$, then fixed in Bouin's solution, with subsequent changes at least until decalcification was complete. Parafin sections (7–9 μm thick) were stained with Delafield's hematoxylin and counterstained with eosin-y and orange-g. Six sagittal sections regularly spaced from the pallial groove to the center of the animal were prepared from each specimen. Oocyte size frequencies were then calculated by measuring only those oocytes sectioned through the nucleus (Pearse and Giese, 1966).

To examine the relationship of testis and ovary in hermaphrodites, the above methods proved inadequate. Instead, the gonads from some additional animals were dissected intact into 1.9% glutaraldehyde in 0.2 *M* Millonig's phosphate buffer (pH 7.6) at room temperature for one hour. The tissue was then rinsed in 2.5% sodium bicarbonate buffer (pH 7.2) for fifteen minutes and transferred to 2% osmium tetraoxide in 1.25% sodium bicarbonate buffer (pH 7.2) for an additional hour, dehydrated in an ethanol series. Three changes of propylene oxide were made before embedding in Medcast resin (Pelco). Sections (0.5-μm thick) were stained with Rich-

Table I

Summary of primary study sites and geographic range in six Lepidochitona *spp.*[1]

Species	Primary study sites (Figs. 1–2)	Known range limits (N to S)
L. dentiens	SJ, BD, BH, PG AN, ST, PP, BC	Alaska–so. California
L. hartwegii	PP, CY, SN, PV, BR	so. Oregon–Baja California
L. berryana	AN, CP	AN–PV
L. thomasi	BC	PP–Mill Creek (near BC)
L. caverna	SC1–3, PP, CY, SB	SC–Dolan Creek (near BC)
L. fernaldi	All Fig. 2 sites	Barclay Sound, B.C.–so. Oregon

[1] See Figures 1 and 2 for symbol key.

ardson's stain (Richardson *et al.*, 1960) and photographed with Nomarski optics.

The brooding species were collected or observed in the field in much greater detail than the free-spawning species. *L. caverna* was sampled for brooding in the field between January, 1980, and June, 1981, and again in March, 1985. *L. thomasi* was sampled between June, 1980 and August, 1982, and in March and October, 1985. *L. fernaldi* was sampled in January and September, 1981, once in August, 1982, and many times during spring, 1983, and fall, 1984, to summer, 1985.

Several methods of maintaining live animals were tried. A system of continuously splashing seawater provided the best health and survival. Large PVC or plexiglass tanks were employed either outdoors with shading, or indoors in a room with good exposure to sunlight. With too little light the chitons starve, and with too much light the substratum is overgrown by algae. To provide continuous splash, seawater lines were connected to a horizontal grid of one-half inch PVC pipes spaced approximately 20 cm apart and drilled on the bottom surface of each pipe were holes, 0.156 cm (5/32″) in diameter, approximately 2 cm apart. This grid was suspended about 20 cm above containers inhabited by chitons.

To isolate one or more chiton(s) from all other chitons, the chiton(s) was allowed to attach to a single rock (or brick) and then placed, surrounded by sand, in two-liter plastic containers approximately two-thirds full of sand. Holes in the plastic containers just above sand level permitted water outflow. This system provided low-maintenance rearing conditions for the adult chiton(s) and any brooded offspring. If no isolation was desired, chitons were allowed to mingle on a continuous hard substratum. When an animal was detached to be checked for brooding activity, there was greatly improved reattachment to hard surfaces if sand and other debris on the foot or in the pallial region were gently brushed away using wood fibers from a broken dry applicator stick. Broods were removed from the pallial region in the same manner.

Broods collected in the field or lab were usually kept in static filtered seawater cultures in small beakers with daily changes of filtered seawater. The progress of many cultures was recorded daily, with an approximate estimate of the percentage of eggs/embryos developing normally, and a recording of the appearance of the most useful developmental features. These included: early cleavage (2–64 cell stage), gastrulation and persistence of the blastopore opening, appearance of beating prototrochal cilia, appearance of eyespots, hatching, and metamorphosis. Although other potentially useful features exist (*e.g.*, foot, valve rudiments, girdle spicules), they were not generally required to determine embryonic stage of development. Some broods were collected during early cleavage and were followed through hatching and metamorphosis. The developmental schedules generated from these broods were used to assign approximate ages of appearance to the above developmental features. With this approach, it was possible to estimate to within approximately one day when any brood was spawned, even those collected at a late stage.

Postmetamorphic juveniles adhering to culture containers were introduced to outdoor splash tanks, preferably positioned away from direct spray exposure. Successful rearing to adulthood by this system required a dependable source of splash, moderately strong light source, seawater with a low level of silt, and removal of filamentous plants that tended to overgrow surfaces, by hand or by addition of grazers (*i.e., Littorina* spp. or the chiton, *Nuttallina californica*). An even higher survival rate for juveniles was attained when adult brooders kept in splash tanks were not disturbed, or if cultured larvae were allowed to metamorphose on the dorsal surface of their parent or on a small piece of barnacle plate and then introduced to a splash environment.

The early development of both brooders and free spawners was compared using light and scanning electron microscopy. All egg diameters were measured from light microscope preparations and from photomicrographs of spawned gametes. Light photomicroscopy with flash illumination as described in Eernisse (1984) was also useful for measuring active larvae. Eggs photographed with SEM were dissected from animals with mature gonads. For SEM, eggs and larvae were rinsed several times in millipore-filtered seawater, pipeted into capsules covered at each end with fine mesh nylon netting to permit fluid exchange, and immersed in 1% osmic acid in seawater buffered with 0.1 *M* sodium cacodylate. This buffer partially avoided dissolving calcareous spicules on the larvae or juveniles during fixation. After fixation, the specimens were prepared for SEM using standard procedures and scanned at 10 or 15 kV on a Nanolab 7™ SEM.

Results

The reproductive observations reported here reveal that species differ in their mode of spawning—either free spawning or brooding—and that among the species that brood there is a difference in mode of fertilization, they are either cross- or self-fertilizing. The evidence for distinctions in reproductive mode, size, and early developmental patterns is presented below, with slightly greater emphasis given to species that brood.

Evidence for free spawning in four species

Free spawning was observed in the lab for *L. dentiens, L. hartwegii,* and *L. berryana.* For *L. dentiens* from central California (BH in Fig. 1), 37 individuals (out of 220 total observed) free spawned on 11 separate occasions from January, 1980, to May, 1981. All 11 spawnings occurred between the months of February and May 1980 and 1981. The histological analysis of 16 females collected at BH during peak spawning periods gave little indication that oocyte size reaches a maximum at any particular sample time; mature and immature eggs were present throughout February to May. The mean oocyte size of these six samples (n = 882 oocytes total) ranged from 38 to 56 μm in diameter, and only about 1.5% of the oocytes counted were large (>100 μm). The oocyte sizes in each of the 6 samples had similar ranges, usually about 8 to 168 μm in diameter (minimum range per individual = 8–152 μm). These spawning and preliminary oocyte size data, and several subsequent observations of spawning in Washington (SJ) populations, suggest that *L. dentiens* is reproductively active at least through winter and spring in both California and Washington.

Individuals of *L. hartwegii* (from PP) were observed free spawning on three separate occasions; in February, March (epidemic spawning), and October. Spawning in *L. berryana* (from SQ) was observed three times; twice in May and once in June. In the first May spawning, three *L. dentiens* males from BH were in close proximity to a *L. berryana* female who subsequently spawned. Care was taken to avoid excessive sperm and possible polyspermy by placing spawning males in another container. At the same time, eight additional individuals (4 males, 4 females) of *L. dentiens* from SJ began spawning in a separate container. Although there was ample *L. dentiens'* sperm available in both cases, only the *L. dentiens* eggs were fertilized, suggesting a block to hybridization between *L. dentiens* and *L. berryana.*

Though free spawning was not observed in the field, the laboratory observations and the absence of brooding was taken as indirect evidence that *L. dentiens, L. hartwegii,* and *L. berryana* were free spawners under field conditions. None of over 1000 specimens I have examined in more than five years were brooding. Collections were frequent enough that it is highly unlikely that brooding of brief duration was missed. A fourth species, *L. keepiana,* has been collected extensively in southern California by others but has never been reported as brooding. I have examined about 200 individuals at Palos Verdes, California (January, 1983) and over 100 individuals at Cayucos, California (April, 1984 and March, 1986). None were brooding.

Brooding in three species

Figures 3a–c depict the seasonal patterns of brooding in *L. fernaldi, L. thomasi,* and *L. caverna.* All three species brood for much or all of the year. Data from several years have been combined because the patterns appeared essentially the same from year to year.

L. fernaldi (Fig. 3a) and *L. thomasi* (Fig. 3b) have lengthy but somewhat restricted seasons, particularly with brooding activity peaking during winter and spring and little or no brooding in the field observed during August to October for either species. During this latter period, less than 1% of all *L. fernaldi* adults observed were brooding (1 of 287 at SJ; 2 of 157 at TN). Likewise, less than 1% of *L. thomasi* adults observed were brooding (0 of 108 at BC; 1 of 112 at other sites on Big Sur coastline). Only occasional brooders of either species were noted in the lab during August to October.

In contrast to *L. fernaldi* and *L. thomasi, L. caverna* may at least potentially brood throughout the year, a much longer reproductive season than previously re-

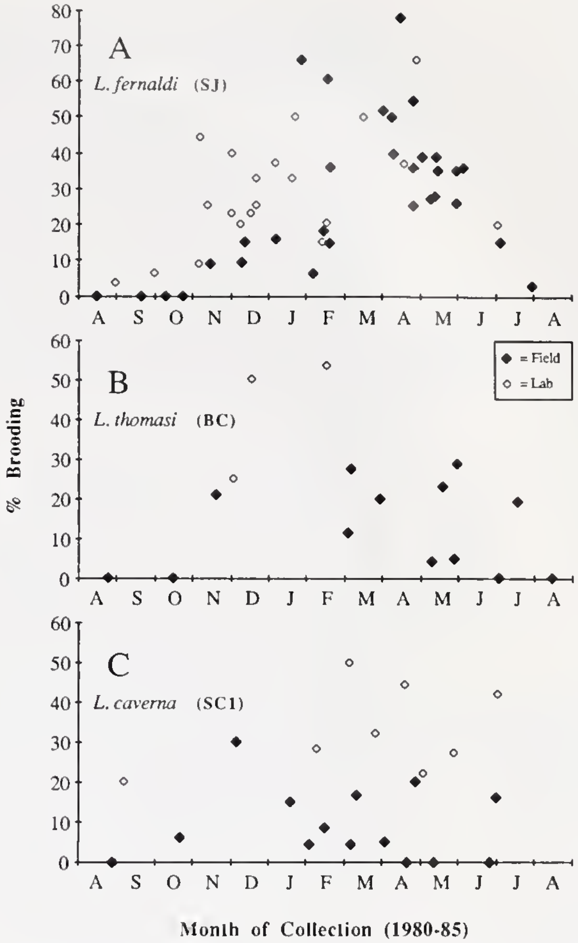

Figure 3. Seasonal brooding activity in *Lepidochitona fernaldi, L. thomasi* and *L. caverna,* between 1980–85. Dark symbols indicate field observations, open symbols indicate lab observations of recently collected animals. Statistics on field collections are given below as: n_c; n_b; n_t; $\bar{x}_b \pm s(n_{min} - n_{max})$; $\bar{x}_t \pm s(n_{min} - n_{max})$, where n_c = # of collections; n_b = total # of brooders; n_t = total # of adults observed; $\bar{x}_b \pm s(n_{min} - n_{max})$ = mean # of brooders per collection ± S.D. (range); $\bar{x}_t \pm s(n_{min} - n_{max})$ = mean # of adults observed per collection ± S.D. (range). *L. fernaldi:* 29; 163; 939; 5.6 ± 3.3 (0–12); 32.4 ± 33.7 (9–196). *L. thomasi:* 13; 79; 419; 6.6 ± 8.7 (0–31); 34.9 ± 27.2 (13–114). *L. caverna:* 14; 36; 457; 2.8 ± 2.7 (0–7); 33.7 ± 12.6 (10–50). Collection sites (see Fig. 1) are SJ, BC, and SC-1 for Figures 3a, b and c, respectively.

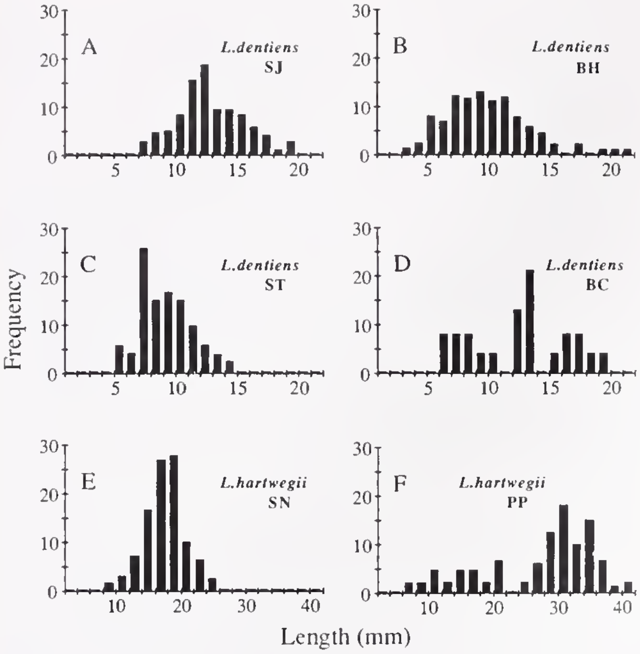

Figure 4. Size frequencies in *Lepidochitona dentiens* and *L. hartwegii* as length in mm, assigned to the nearest whole mm size class (see text). Sample statistics (given as n; $\bar{x}$ ± S.D.; x_{max}) are as follows: (4a) 145; 12.5 ± 3.2; 27.3; (4b) 304; 9.4 ± 3.3; 22.0; (4c) 55; 8.7 ± 2.1; 14.0; (4d) 23; 12.2 ± 4.0; 18.5; (4e) 105; 16.1 ± 3.0; 23.6; (4f) 50; 25.8 ± 8.6; 39.7. Site abbreviations explained in Figure 1 legend.

ported by Heath (1907) (as *T. raymondi*). For unknown reasons in samples spread throughout the year, a high percentage of recently collected animals began brooding when brought into the lab, even during periods when no animals were observed brooding in the field (Fig. 3c—open symbols).

Size

The *Lepidochitona* considered here consist of five species with small adults and one, *L. hartwegii*, with medium-size adults (Figs. 4, 5). Among the brooding species, *L. thomasi* is largest but is still small compared to other chiton species. In the populations sampled, *L. thomasi* is characterized by a significantly greater mean length than *L. caverna* and *L. fernaldi* (Student *t*-test; $P < 0.001$ in both cases), and also has a significantly larger minimum brooding size ($P < 0.001$ in both cases). Smaller maximum adult size in *L. caverna* and *L. fernaldi* thus corresponds to smaller size at first reproduction. For each of the brooding populations, there is no apparent change in frequency of brooding with increasing size.

The size characteristics of a species vary significantly among populations, including comparisons of *L. hartwegii* (Fig. 4e, f), *L. dentiens* (Figs. 4a–d), and *L. caverna* (Figs. 5c, d) ($P < 0.001$ in each case). These size differences between populations were consistent throughout the year. Observed size characteristics of a population should therefore not be equated with those for a species throughout its geographic range.

Comparisons of early development

The eggs of *L. thomasi* and *L. fernaldi* are largest of the six species (260–280 μm diameter). Those of *L. caverna* and *L. berryana* are intermediate (220–240 μm diameter). Those of *L. dentiens* and *L. hartwegii* are smallest (200–220 μm diameter). Similarly, the larvae and recently metamorphosed juveniles become progressively smaller. These results match expectations that brooders normally have larger egg/larval size than closely-related free spawners, but the difference is not nearly as pronounced as in the case of most previous brooders and free spawners contrasted (see Discussion).

The eggs also differ in the ornate sculpturing of the transparent, extracellular egg hulls, illustrated here as imaged with SEM (Figs. 6a–f). With the exception of *L. thomasi* and *L. fernaldi*, whose egg hull sculpturing appears virtually identical (Figs. 6e, f), each species can be

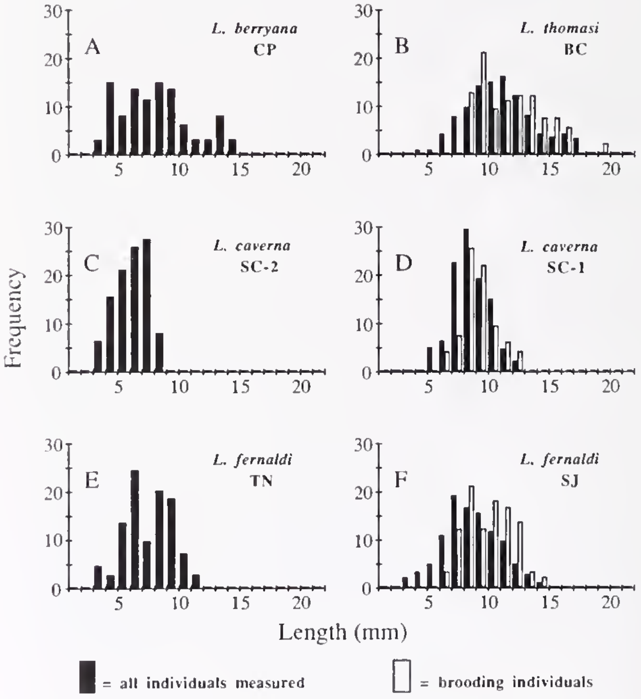

Figure 5. Size frequencies in *Lepidochitona berryana, L. thomasi, L. caverna,* and *L. fernaldi* as length in mm, assigned to the nearest whole mm size class (see text). Sample statistics (given as in Fig. 4 legend) are as follows: (5a) 38; 7.6 ± 2.9; 14.1; (5b) all individuals: 341; 10.4 ± 2.7; 18.5; brooders only: 57; 11.3 ± 3.1; 18.5; (5c) 68; 5.7 ± 1.3; 8.4; (5d) all individuals: 147; 8.2 ± 1.4; 12.0; brooders only: 42; 8.6 ± 1.4; 12.0; (5e) 45; 7.1 ± 1.8; 10.9; (5f) all individuals: 355; 8.3 ± 2.4; 17.0; brooders only: 89; 8.7 ± 2.2; 15.2. Site abbreviations explained in Figure 1 legend.

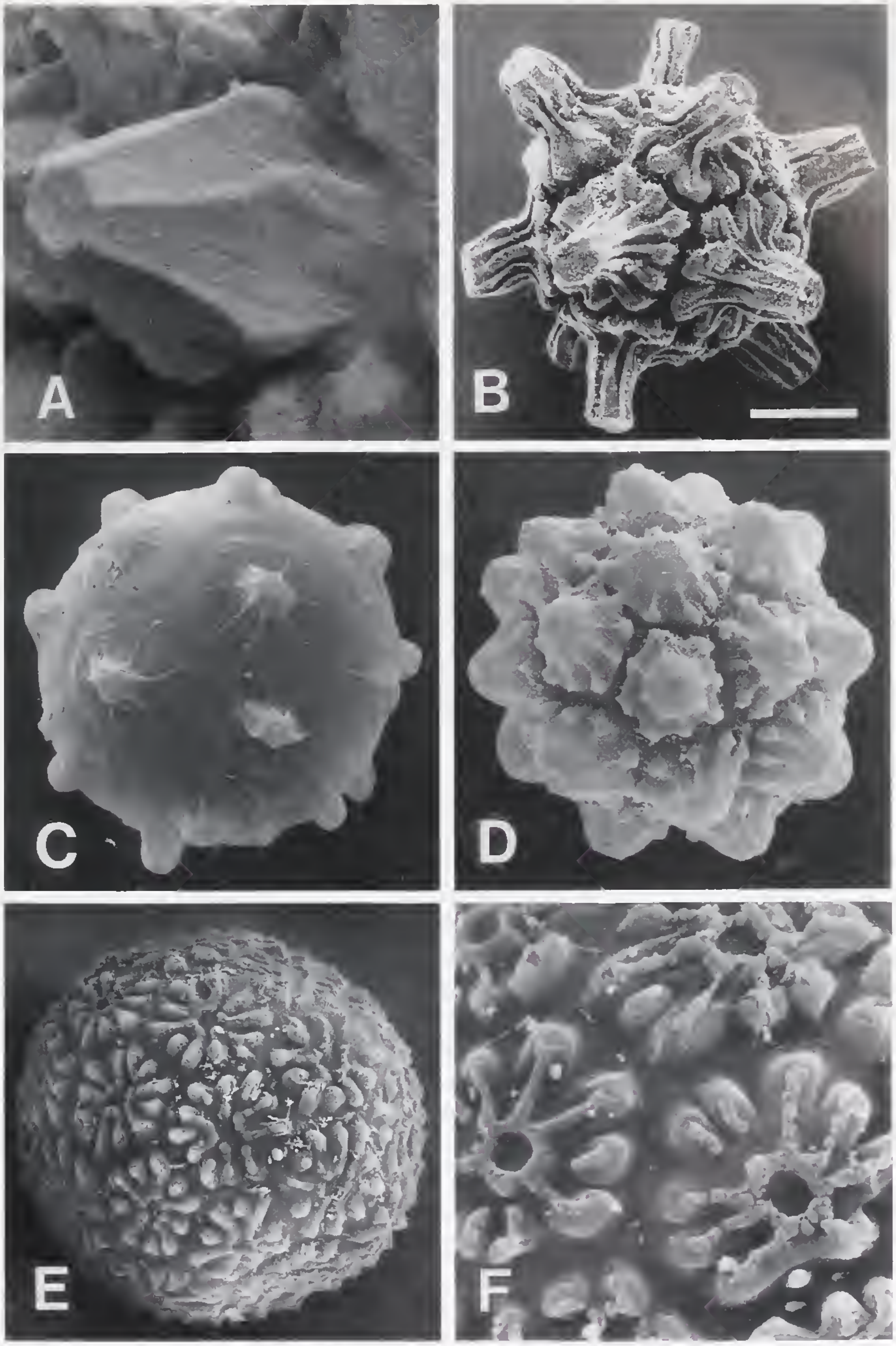

Figure 6. Eggs of six species of *Lepidochitona* as imaged with SEM. All eggs were dissected from animals with mature gonads but, except for a slightly smaller egg diameter, appear very similar to eggs that are spawned. The eggs correspond to (a) close-up of *L. hartwegii* egg hull; (b) *L. berryana;* (c) *L. caverna;* (d) *L. dentiens;* (e) *L. thomasi;* and (f) close-up of *L. fernaldi* egg hull surface. Scale bar: (a, f) = 20 μm, (b) = 63 μm, (c) = 48 μm, (d) = 54 μm, and (e) = 51 μm.

distinguished by its characteristic pattern of sculpturing. The free spawners (Figs. 6a, b, d) also have much more elongate cone-shaped hulls than the brooders (Figs. 6c, e, f).

In all three free-spawning species, the larvae at the time of hatching are very similar and can be represented by the SEM image of a two-day-old *L. hartwegii* trochophore in Figure 7a. At this stage, the larvae swam continuously and lacked a foot for attachment. In cultures with a high (>90%) fertilization success, very little variation

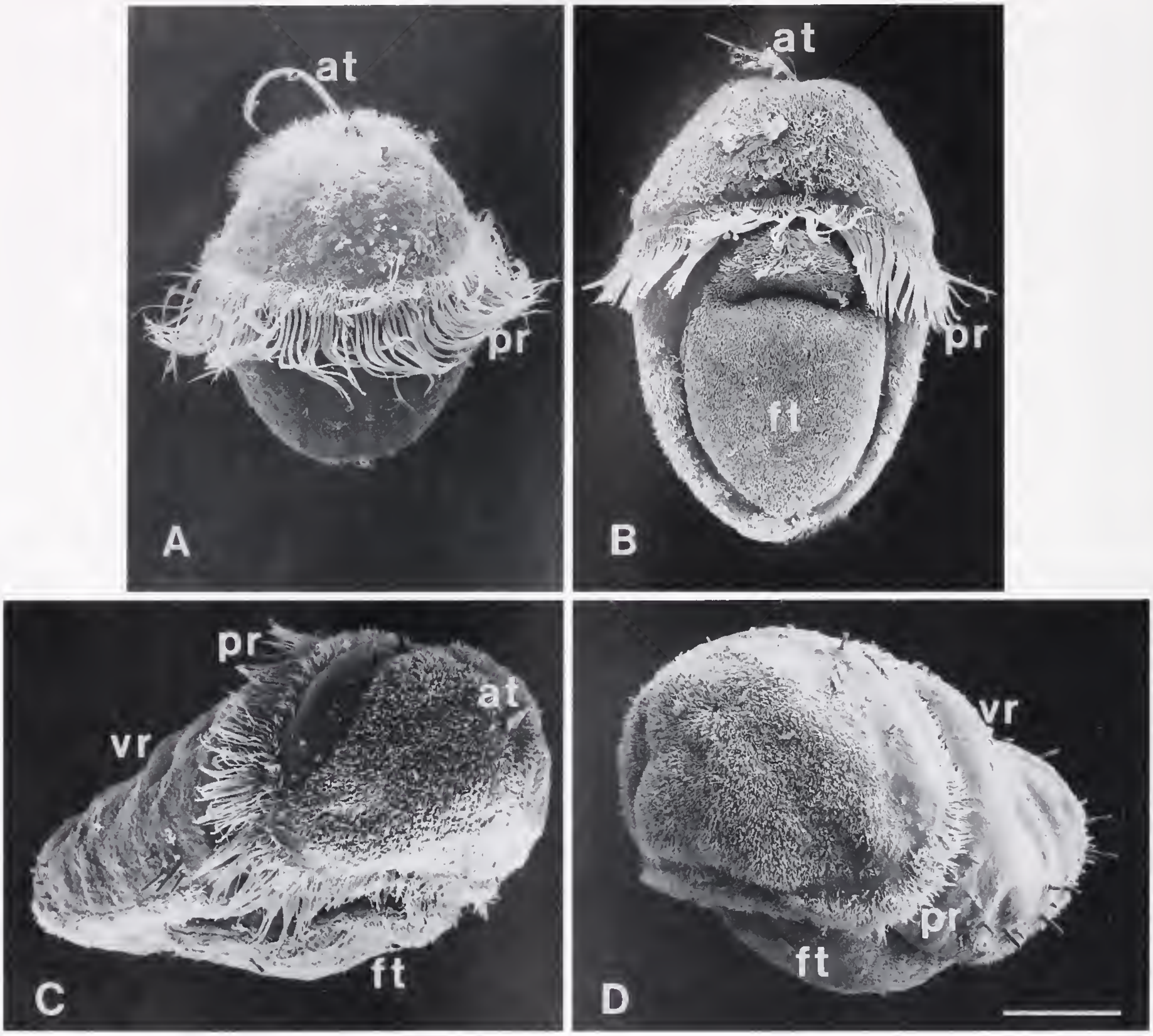

Figure 7. Larvae near stage of hatching as imaged with SEM in (a) *Lepidochitona hartwegii* (approx. 2 days old, fixed approximately 12 hours after synchronous hatching of a culture from one free-spawning female); (b) *L. caverna* (approx. 7 days old); *L. thomasi* (approx. 9 days old); and (d) *L. fernaldi* (approx. 9 days old). Explanation of symbols: pr = prototroch; at = apical tuft; ft = foot; vr = valve rudiments. Scale bar: (a–d) = 50 μm.

in time of hatching was observed in any of the species. In several cultures observed in the most detail, about 80 to 90% of the larvae hatched within a few hours of each other. A less-precise estimate is possible for all other cultures which were monitored only daily. For these, I conclude that more than 95% of the embryos hatched between 24 and 48 hours (13–16°C), if they hatched at all.

Larvae from free-spawning species swam continuously in culture for several days, until the foot and eyespots developed. These larvae began exploratory creeping behavior at about 5–6 days (at 13–16°C). Some larvae metamorphosed at about 8–10 days. However, most larvae did not metamorphose, and there was substantial variation in size of juveniles that did metamorphose. Most hatched larvae either deteriorated or remained unmetamorphosed for weeks after other larvae in the same culture had completed metamorphosis. I did not investigate stimuli to metamorphosis in these species.

In contrast, the embryos of all three brooding species hatched as larvae (*i.e.,* still with a prototroch) but at an advanced stage ready to metamorphose. Unlike recently hatched free-spawned larvae, brooded larvae already have a well-developed foot by the time they hatch (Fig. 7b–d), and thus are capable of creeping and consequently can at least potentially remain in the vicinity of their parent(s) and siblings. Brooded larvae hatch about seven to eleven days after fertilization at 14–16°C, and thus hatch much later than do embryos of free spawners. The eyespots (a convenient unambiguous indicator of larval stage) appear about four to five days after fertilization—well after all the free-spawned larvae have hatched and well before brooded larvae hatch.

Hatching stage was influenced by the removal from the brooding parent. Broods removed from an adult at a very late stage of development often began hatching immediately, with most embryos hatching within one or two hours. Such emerging larvae could be one to two days more developed at hatching than larvae shown in Figures 7b–d. These larvae could complete metamorphosis within a day of hatching. These observations suggest that artificial culturing of broods stimulates a precocious hatching of embryos. Thus, embryos brooded naturally are even more likely to avoid pelagic dispersal upon hatching.

Larval morphology and development noticeably differs among brooders. Cultured embryos of *L. caverna* are slightly smaller and hatch earliest, roughly seven to nine days after fertilization at 14–16°C (Fig. 7b). At this stage, the larvae normally swim for one to two days in static cultures before settling and metamorphosing (defined as loss of prototroch; Pearse, 1979). Swimming, however, may be stimulated by artificial culturing, since juveniles soon appear on or near undisturbed brooders isolated in the lab (see below). Cultured embryos from both *L. thomasi* and *L. fernaldi* hatch slightly older and larger than those of *L. caverna,* about nine to eleven days after fertilization at 14–16°C (Figs. 7c, d). *L. fernaldi* embryos develop only slightly slower when cultured at the 8–12°C they normally experience when submerged at high tide (hatching by 12 days at 10°C). Again, metamorphosis generally occurs within two days of hatching in static cultures. In both *L. fernaldi* and especially *L. thomasi,* brooded larvae are sluggish when they hatch, preferring crawling to swimming. *L. fernaldi* larvae differ from *L. thomasi* (and *L. caverna*) larvae in consistently lacking an apical tuft, and possessing shorter prototrochal cilia (Fig. 7d).

Egg clusters of brooders also differ among species. The color of the egg cluster varies according to stage of development. Coloration proceeds from greenish to golden in *L. caverna,* from dark brownish green to tan in *L. thomasi,* and from light brown to golden brown in *L. fernaldi.* In *L. caverna,* the eggs are held loosely. In both *L. thomasi* and *L. fernaldi* the eggs are held together with mucus and two (or one) rod-like egg clusters can be removed intact from the brooder. Adults of these latter species are much more mobile than adults of *L. caverna* during brooding; the tightly clustered broods may permit increased mobility and feeding by adults.

In contrast to the free spawned larvae, larvae from brooders metamorphosed reliably in the lab, even in culture vessels filled only with filtered seawater. Survival was usually high (>95%) and morphological changes immediately following metamorphosis were apparent, especially a marked change in body outline as elongate larvae become oval-shaped juveniles. Girdle spicules proliferated and calcification of the valves was initiated or at least greatly accelerated. In containers with a film of microorganisms, but without overgrowth of filamentous diatoms, newly metamorphosed juveniles were observed to feed actively, leaving a trail of marks from radular scraping as they crawled (Eernisse and Kerth, 1988). Growth was generally rapid, indicating that these juveniles were developing normally.

Evidence for hermaphroditism and self-fertilization in L. caverna

Heath (1907) briefly described a simultaneous hermaphroditic condition in *L. caverna* (as *T. raymondi*). I also found that typical *L. caverna* are hermaphroditic and, as noted by Heath, have a large majority of their gonad occupied by ovaries. Figure 8 shows the intact gonad of one of two adult animals, raised in the lab, that were dissected and fixed in September, 1986. The testicular lobules in this animal are restricted to the peripheral portions of the gonad, and several contain mature sperm. This gonad is consistent with the observed gonad of more extensive field samples of *L. caverna* from 1980 to 1986. Sperm (and eggs) were regularly observed in wet dissections of live adults, and had a shape and size similar to other chiton sperm, although they were never abundant. Histological samples of 110 animals, from collections of about ten animals taken every six weeks between February, 1980 and June, 1981, showed most animals had mature gonads, regardless of the season, primarily filled with eggs. Of 94 animals sampled with detectable gonads, 89 possessed eggs. Only five males lacking eggs were found. Sixteen specimens could not be sexed either because they were reproductively immature, or because the sections were inadequate. Small amounts of sperm or testes were seen in many of these 89 "females," similar to those in Figure 8, but were not found in others, probably because of their sparseness or because of incomplete infiltration of fixative and embedding medium as indicated by open spaces in these whole mount sections.

To test the potential ability of *L. caverna* to self-fertilize broods, I repeated, on a more limited scale, the isolation experiments of Pearse and Lindberg (1980), also as in their studies (reported as *C. dentiens*) at Long Marine Laboratory with animals collected from a nearby, Santa Cruz, sea cave (the type locality). Five individuals each produced cohorts of offspring more than one month after isolation, including some juveniles that survived to adult sizes.

Evidence for hermaphroditism and self-fertilization in L. fernaldi

When I first discovered *L. fernaldi* populations in Washington, I identified them as northern populations of the morphologically similar gonochoric species, *L. thomasi*, known previously from only a few localities in central California (Eernisse, 1986). An experiment initially designed to test the hypothesis that individuals from two geographically isolated populations could be crossbred in the lab, instead became a comparison of the capacity of *L. fernaldi* and *L. thomasi* to breed without conspecific mates.

Eight pairs, each consisting of one BC *L. thomasi* known to be a female and one randomly selected SJ *L. fernaldi*, were isolated on 8 February 1981; each pair was independently supplied with splashing seawater. Neither species is known from the vicinity of Santa Cruz, so it is highly unlikely that sperm could have entered through the Long Marine Lab seawater system. Nearly all the *L. fernaldi* individuals began brooding, and broods were fertilized; larvae hatched and metamorphosed normally. This suggested that the *L. fernaldi* brooders were hermaphrodites capable of self-fertilizing their broods. In contrast, some of the known *L. thomasi* females began brooding, but the broods failed to develop (see below).

New experiments were then performed with 32 *L. fernaldi* individuals isolated completely from all other individuals, and periodically sampled for broods. As before, animals produced a large percentage of fertilized eggs. Of 25 individuals surviving in isolation for more than 60 days, 16 produced at least one brood and nine produced multiple broods. Altogether, I collected 31 broods, including 20 that were more than 90% fertilized. Of the remaining 11, two were less than 25% fertilized and two more appeared fertilized but succumbed to bacterial infections. The large majority of embryos hatched and metamorphosed successfully in culture.

Of the individuals spawning multiple broods, one spawned five separate broods, each at least partially fertilized. I collected broods from this individual, isolated from 25 October 1981 to 13 June 1982 (231 days), on days 41, 60, 76, 88, and 195. Coincidentally, the first four broods were discovered at approximately the same advanced stage of development, so this animal was spawning broods at approximately two week intervals. Three individuals brooded three times each; again each brood was at least partially fertilized with six of the nine broods more than 95% fertilized. One of these nine broods (>50% fertilized) was collected 274 days (approx. nine months) after the animal was isolated. For all seven individuals that produced multiple broods, I detected no significant difference (Mann-Whitney U Statistic) between the mean percent fertilization of the first ($\bar{x} = 78.1$ %; S.D. = 33.9) and last ($\bar{x} = 80.0$; S.D. = 36.6) brood.

Approximately 25 live *L. fernaldi* were, at various times, dissected without finding any evidence of spermatophores. These dissections, combined with later histological analyses of five animals, suggest that normally only about one out of every 10 individuals produces large testes and no obvious ovaries, while most if not all other individuals possess mostly ovaries and very small amounts of testes. The *L. fernaldi* gonads appear similar to those previously described for *L. caverna* (Heath, 1907; this study). Simultaneous hermaphroditism and reproduction by isolated individuals suggests strongly that *L. fernaldi* is capable of self-fertilization, although it does not completely rule out the possibility of parthenogenesis (see Discussion).

Evidence for strict gonochorism in L. thomasi

Histological sex determination provided evidence that *L. thomasi* is normally gonochoric, since strict males were relatively common. Three separate collections were made at BC during a period of known brooding activity. Sex was determined from sections. Of 27 individuals sectioned, 9 were males with large testes, 5 were females without any trace of sperm, and 13 could not be sexed either because their gonads were immature or because the sections were inadequate. It is likely that this male-biased sex ratio resulted from a small sample size, although some male-biased inequalities have been observed for chitons (Pearse, 1979; Sakker, 1986). An additional, unquantified impression that males are at least as common as females comes from the experience of dissecting over 150 frozen or live *L. thomasi* for electrophoretic studies, including animals from seven localities on the Big Sur coastline collected over several years.

Based on morphological and electrophoretic affinities relative to other members of the genus, I previously argued that *L. fernaldi* and *L. thomasi* are closely related (Eernisse, 1984, 1986). The great similarity in egg hull sculpturing patterns presented here (Fig. 6) further corroborates this conclusion. Yet these species are, without doubt, morphologically and electrophoretically distinct from each other. Moreover, I show here that *L. fernaldi* is hermaphroditic, whereas *L. thomasi* individuals are either female or male, and females do not produce fertilized broods in isolation. Twenty-seven isolated individuals of *L. thomasi* were sampled for brooding periodically while under isolation. Although the observations were at least as frequent as for *L. fernaldi*, only eight broods were collected and none of these broods developed normally.

To be certain that this failure was due to lack of fertilization I isolated eight *L. thomasi* females known to have

Table II

Summary of reproductive patterns in six Lepidochitona *spp.*

Species	Spawning mode	Inferred fertilization mode	Mean egg size	Mean age at hatching	Max. adult length
L. dentiens	free spawner	cross	210 μm	36 h	27.3 mm
L. hartwegii	free spawner	cross	210 μm	36 h	45.1 mm
L. berryana	free spawner	cross	230 μm	36 h	14.1 mm
L. thomasi	brooder	cross	270 μm	8 days	18.5 mm
L. caverna	brooder	self	230 μm	10 days	13.9 mm
L. fernaldi	brooder	self	270 μm	12 days	17.0 mm

previously brooded, and checked daily for freshly spawned broods. During one month I collected broods from three individuals. Immediately after each collection, I dissected a male *L. thomasi*, and used its sperm to inseminate a portion of the collected eggs. Another portion of eggs was kept without sperm as a control. Each of the three times this experiment was performed at least some of the eggs with sperm added became fertilized (20, 60, and 100%), but none of the eggs without added sperm were fertilized. This demonstrates that spawned eggs are potentially viable, but do not become fertilized because of lack of viable sperm.

Discussion

The results of life history comparisons among six northeastern Pacific species of *Lepidochitona* are summarized in Table II. Similarities among the *Lepidochitona* considered here include the size and rates of development of embryos. Eggs of brooders are roughly the same or slightly larger than eggs of free spawners, and the size of newly metamorphosed juveniles of brooders and free spawners is correspondingly similar. Differences between free spawners and brooders include stage (*i.e.*, age) at which embryos hatch. Stage at hatching appears to be a relatively inflexible character within a particular species. I have presented evidence that this relatively minor developmental difference between free spawners and brooders has a functionally important consequence: brooded offspring, but not free-spawned offspring, at least have the potential to crawl away. Here I argue that a potential to remain near one's parent may explain some, but not necessarily all, of the life history traits shared by *Lepidochitona* brooders.

Most previous studies contrasting brooding and free spawning marine invertebrate species (*e.g.*, Menge, 1975) have reported dramatic differences in egg size and larval feeding between brooders and free spawners. Although the correlation of these factors with mode of spawning is an interesting subject in its own right, these differences confound interpretations concerning the consequences of crawl away dispersal that can result from brooding. Brooders typically have relatively few, large yolky eggs with lecithotropthic development, while free spawners have numerous small eggs with lengthy planktotrophic development. The comparison of brooders and free spawners in *Lepidochitona* appears less complicated by larval feeding or egg size. Specifically, planktotrophy is unknown for chitons, so the comparison of brooding and free spawning chitons is uncomplicated by a selective advantage that might be gained from larval feeding (Grant, 1983). Furthermore, although *Lepidochitona* brooders in some cases have larger eggs than free spawners, as much a two-fold volume difference, previous studies have typically reported egg diameters which translate to a five- to ten-fold egg volume difference (*e.g.*, Menge, 1975). Thus, the larval size, morphology, and feeding ability differ to a lesser degree in *Lepidochitona* than in most previous comparisons of brooders and free spawners.

The differing potential for emerging larvae to crawl away in *Lepidochitona* is repeated within chitons as a whole. *L. dentiens*, *L. hartwegii*, and *L. berryana* are like other free-spawning chitons (Pearse, 1979; Strathmann and Eernisse, 1987) whose pelagic embryos invariably hatch out of their egg capsules soon after their prototrochal cilia become active. In a literature search of over 50 species of chitons reported to spawn eggs freely or in loose strands of mucus, I could find no exceptions to this pattern. Free spawned embryos hatch several days before a foot has developed, so that even if embryos were retained until hatching, they would presumably have difficulty remaining attached to a substrate. The active swimming observed in laboratory cultures of free spawners also suggests they are normally pelagic.

In contrast, *L. caverna*, *L. fernaldi*, and *L. thomasi* are like other chiton brooders, retaining embryos in the paired pallial grooves to an advanced stage of development. Brooding is known with certainty for about 30 (out of about 800 total) chiton species as compiled by Pearse (1979) and added to by Sirenko (1973), Penprase (1979), O'Neill (1984), Burn (1984), Cochran (1986), Creese (1986), Kaas and Strack (1986), Creese and O'Neill (1987), and the present study. Additionally, there are at least a few chiton species that neither free spawn nor brood. Instead, they embed their eggs in a benthic mass of jelly-like substance, and leave this egg mass behind, often attached to rocks or algae (Heath, 1899, 1905; Risbec, 1946; Matthews, 1956). All studied chiton brooders or benthic egg mass layers hatch as larvae sufficiently advanced so that they are already endowed with a fully functioning foot for crawling. Embryos from most or all of these species hatch shortly before metamorphosis, although in some of the above cited cases, offspring may remain in the pallial groove until well after metamorphosis.

I do not claim that brooders are without variation.

Both inter- and intraspecific differences among brooders in stage of hatching suggests that there may be some variation in facultative planktonic dispersal ability of brooders. Still, the observed variation in chiton brooders is slight enough so that even the earliest hatching brooded larvae have a good opportunity to remain near parents. For example, of the three *Lepidochitona* brooders considered here, *L. caverna* is exceptional because it hatches at a "free swimming" stage when embryos are cultured apart from their parent. Similarly, other chiton brooders demonstrate flexibility in swimming behavior, depending on circumstances. For example, I have observed the same "early" hatching stage (*i.e.*, swimming, but with a well-developed foot, eyespots, shell plate rudiments, etc.) in cultured embryos of *Lepidochitona corrugata* (Reeve, 1848), collected from brooders in Yugoslavia (Eernisse, unpub.), in agreement with Kowalevsky's (1883) observations for this species (referred to as *Chiton polii* Philippi, 1836). Even in these chiton brooders that hatch at a "free swimming" stage in culture (*e.g.*, *L. caverna* and *L. corrugata*), there appears to be a clear opportunity for offspring to crawl away.

At another level is potential variation within a species. Creese (1986) recently reported variation in both hatching stage and embryo size in brooders of what he considered to be geographically isolated populations of the same species, *Onithochiton neglectus* de Rochebrune, 1881. I searched for, but did not find, such variation in *L. fernaldi* compared from all sites in Figure 2, or in *L. caverna* from sites SC1-3, PP, CY, or SB. Egg and larval size, as well as time to hatching, appeared approximately uniform within each species. The variation Creese found is unparalleled in my own studies, but even the *O. neglectus* populations he reported as hatching earliest were sufficiently well developed to be capable of crawling away.

Likewise it is not possible to conclude that some chiton brooders never have planktonic dispersal, even for species found brooding fully metamorphosed juveniles. To my knowledge, there are no reports of adults externally brooding *unhatched*, fully metamorphosed juveniles, so I suspect that all chiton brooders may have at least some occasional ability to swim as larvae. An exception might be the possibly ovoviviparous chiton, *Callоplax vivipara* (Plate, 1899), a single animal of which was reported by Plate (1899) to be internally brooding 15 metamorphosed juveniles. In practice, it would be difficult to estimate the degree to which planktonic dispersal is important for a particular brooder.

Differences in spawning and stage at hatching may explain why brooders tend to have a patchy distribution, relative to free spawners. The free spawners that hatch early are typically found wherever suitable habitats are searched throughout their range. *Lepidochitona* free spawners are typically widespread (although not always common) and are more uniformly distributed than the species that brood (Eernisse, 1986). The brooders, whose larvae can crawl away, are found in isolated but locally dense populations. For example, several authors during the last century have expressed exasperation after failing to find *L. thomasi*, even though this brooder is quite abundant locally (Eernisse, 1986). Likewise, the only recently described and rarely collected *L. caverna* and *L. fernaldi* have similar, patchy, distributions. Quantifying the absence of brooders from localities that appear suitable is difficult, but the abundance of brooders where they do occur speaks strongly for their colonization ability. For example, a dramatic colonization event was observed after the recent opening of the Monterey Bay Aquarium, Monterey, California. In July, 1986, within a year of the creation of an artificial, high energy tidepool environment at the aquarium, I observed *L. caverna* to be present in densities estimated at 500 per square meter (Eernisse, unpub.), especially individuals less than 3 mm length. None of the many chiton free spawners of the Monterey Peninsula region were observed in even moderate densities.

Brooders also tend to have a somewhat more restricted geographic range than free spawners, although the range of some brooders is relatively great. For example, the geographic range of *L. fernaldi* extends a considerable distance (many sites in the San Juan Islands, several sites in Puget Sound, on Bordelais Island in Barclay Sound, Vancouver Island, British Columbia, and several sites in southern Oregon) (Eernisse, 1986; this study). In contrast, *L. thomasi* appears to be restricted to the Big Sur coastline and vicinity, a total known distribution stretching only approximately 30 km. Of 137 intertidal invertebrate species surveyed at the Landels-Hill Big Creek Reserve near Big Sur, which had a known geographic range, *L. thomasi* was the invertebrate species with the most restricted distribution (Lindberg, 1984). The rather extensive range of *L. fernaldi* may either indicate that facultative planktonic dispersal has been relatively effective in some cases or that juveniles or adults have rafted successfully, perhaps on algae, as Simpson (1977) first proposed for the widespread subantarctic brooder, *Hemiathrum setulosum* Carpenter, in Dall, 1876. *Lepidochitona* could conceivably be dispersed by rafting; I often observed them in the field on holdfasts and in laboratory cultures on algae such as *Ulva* spp.

Two of three brooding species in *Lepidochitona* are hermaphroditic. *L. thomasi*, and the free spawners, have separate sexes. The first and only previous report of hermaphroditic chitons by Heath (1907) is often cited, but is repeatedly attributed to the wrong species, usually *Trachydermon raymondi* Pilsbry [=*Lepidochitona dentiens* (Gould)] (Eernisse, 1986). Heath (1907) called attention to the simultaneous hermaphroditic condition of small brooding chitons he collected from high tidepools at Pacific Grove, California, commenting briefly on how early and in what form the gonad appears. Figure 8 adds new detail to Heath's description of the hermaphroditic gonad of *L. caverna.* Like Heath, I observed testicular lobules and sperm in the peripheral regions of the gonad, with the great majority of total gonad space occupied by ovaries.

As in predominantly selfing plants which, as a rule, have dispropotionately small amounts of pollen, a small amount of testis relative to ovary in a hermaphroditic animal is usually indicitive of self-fertilization (Charnov, 1982). This possibility is supported by my observations of successful brooding in isolated *L. caverna* and *L. fernaldi,* which excluded the possibility that isolated individuals were outcrossing, provided that long-term sperm storage (up to nine months) was not occurring. For both species, the possibility of sperm storage seems remote based on the simple gonoduct arrangement possessed by chitons and the lack of copulatory structures. Storage for this length of time would seemingly require highly specialized spermatophores, although the prosobranch gastropods, *Urosalpinx cinerea* and *Eupleura caudata,* which copulate but appear to lack discrete spermatophore structures, have been reported to store sperm for periods exceeding one year (Hargis and MacKenzie, 1961). If the possibility of sperm storage is discounted, these isolation experiments provide indirect evidence that these animals are capable of self-fertilization (or parthenogenesis).

Because both hermaphroditic chiton species are brooders, but most brooding chiton species are not hermaphroditic, hermaphroditism is most likely secondary to brooding. Ghiselin (1974) made a similar argument for echinoderms. An association between brooding and hermaphroditism has been noted for a variety of marine invertebrates (reviews by Ghiselin, 1969, 1974; Charnov, 1982; Strathmann and Strathmann, 1982). Strathmann *et al.* (1984) and Eernisse (1984) suggest that when hermaphroditism is found in normally gonochoric groups, such as chitons, it is usually in species that release crawl-away larvae or juveniles. Moreover, these hermaphrodites are typically capable of frequent self-fertilization, an exceptional practice that most hermaphroditic marine invertebrates have elaborate adaptations to avoid. By linking the infrequent occurrence of self-fertilization via hermaphroditism to the potential to crawl away that results from brooding, these authors are postulating that some consequence of limited dispersal must result in conditions favorable for the spread of self-fertilization or, conversely, for the escape from dependence on cross-fertilization.

The mechanism proposed to explain the association of brooding and selfing involves a trade-off between the normally deleterious consequences of inbreeding and the theoretical advantages that departure from outbreeding should entail, all other factors being equal. Strathmann *et al.* (1984) and Eernisse (1984) suggested that brooding (or other types of development with low dispersal) may serve as a precondition to the spread of self-fertilizing hermaphrodites in a population of gonochorists. These authors assumed that occasional hermaphroditic individuals could arise in a normally gonochoric population and, additionally, not always experience a block to self-fertilization. Inbreeding depression would normally reduce the viability of selfed offspring in outcrossing populations, thus usually blocking the success of occasional hermaphrodites. However, if brooding results in prolonged inbreeding, thus exposing and eliminating deleterious alleles, then there may be little additional inbreeding depression if selfing were to occur. If a hermaphrodite were to arise in a highly inbred population and, additionally, had a reduced allocation to male reproduction (*i.e.,* by producing more ovaries than testes) and an ability to self, then it would be expected to gain a reproductive advantage, relative to the cross-fertilizing brooders in the population (Williams, 1979; Charlesworth and Charlesworth, 1981; Charnov, 1982; Bell, 1982). Comparisons of genetic diversity patterns among free spawning and brooding species of *Lepidochitona* (Eernisse, 1984; unpub. data) are at least consistent with this several part hypothesis since gonochoric brooders are less genetically diverse (*i.e.,* possibly more inbred), as detected by allozyme variation, than are free spawners. These results will be considered in subsequent studies, as will the results of paternity analyses using allozyme markers, which support self-fertilization (but do not exclude parthenogenesis) as the normal mode of reproduction in at least *L. fernaldi.*

Another trait linked to brooding may have little to do with limited dispersal. Brooding is linked to small adult body size of chitons in general (Pearse, 1979) and this study documents similar trends among West Coast *Lepidochitona.* All three brooding species are small, generally not exceeding 2 cm in length (Figs. 5b–f). By contrast, the free spawning *L. hartwegii* (Fig. 4f) can exceed four cm in length (Eernisse, 1986). The two smallest *Lepidochitona* species examined here are both brooders. Although the largest brooding species, *L. thomasi,* is as large as some of the free-spawning *Lepidochitona,* this species is certainly small compared to other chiton species in general.

Strathmann and Strathmann (1982) reviewed the association of small adults and brooding in a variety of marine invertebrates and the hypotheses that may explain this correlation. Such hypotheses are not necessarily mutually exclusive, so several factors may be responsible for the observed small adult size of brooding species of *Lepidochitona.* Distinguishing the relative importance of these factors remains to be attempted experimentally, but some hypotheses do not seem likely explanations.

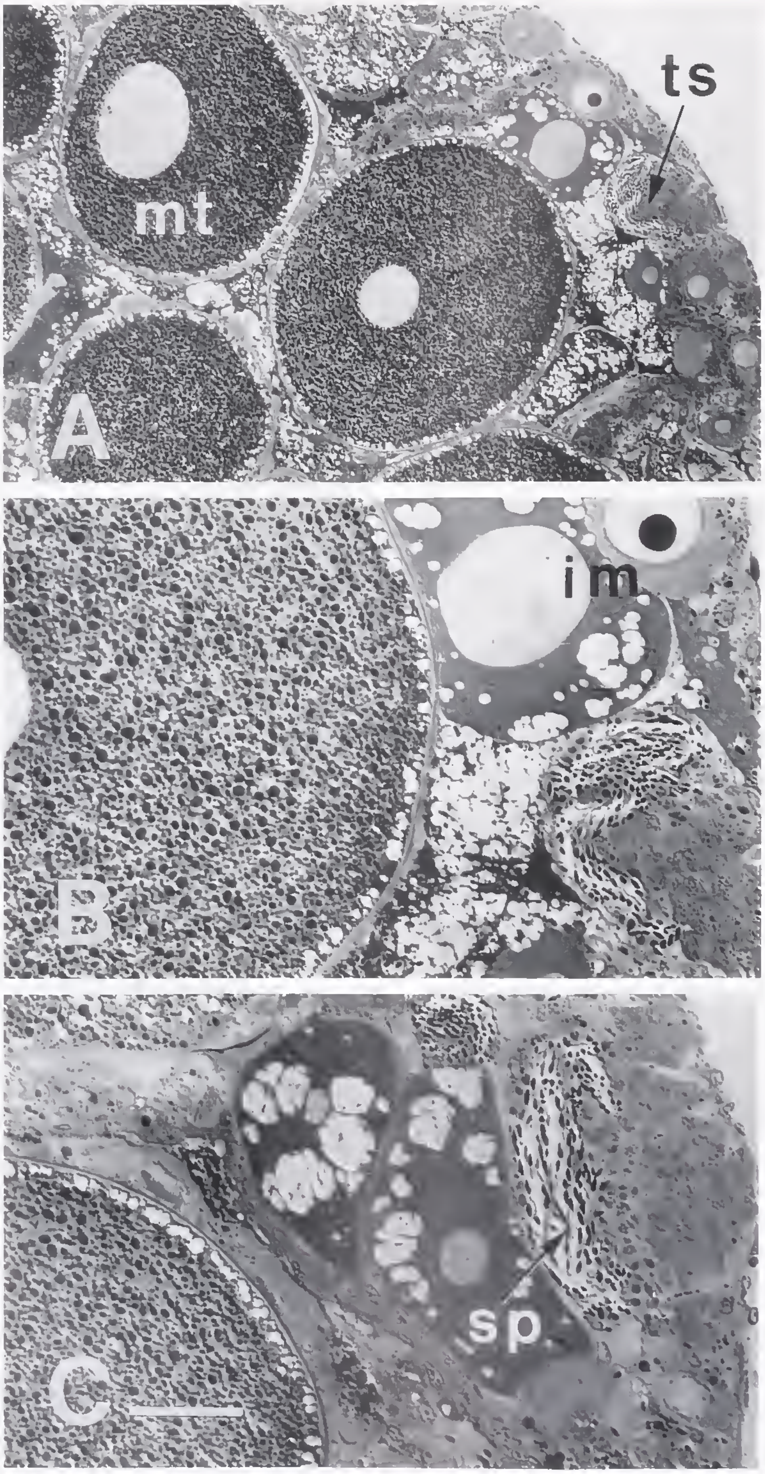

Figure 8. Sections (0.5 μm thick) of the hermaphroditic gonad of one specimen of *L. caverna.* Explanation of symbols: im = immature oocytes; mt = slightly more mature oocytes (still not full size); ts = testicular lobule; sp = mature sperm. Scale bar: (a) = 25 μm; (b, c) = 10 μm.

For example, the suggestion by Christiansen and Fenchel (1979) that the prevalence of brooding in small animals is due to a smaller size at metamorphosis does not hold for *Lepidochitona.* Size at metamorphosis of brooders is equal to, or greater than, size at metamorphosis of nonbrooders.

Another hypothesis, first proposed by Ghiselin (1963) and often repeated, is that animals with small adult body size are limited in energy and thus cannot afford a planktonic stage, which is assumed to depend on high fecundity. Strathmann and Strathmann (1982) consider this an incomplete explanation because it fails to explain why large animals should not brood. I argued above that the most obvious difference between free spawners and brooders was a developmental difference—stage at hatching—which imposed obligatory dispersal for free spawners but not brooders. It is possible that a less-obvious difference may account for the link between brooding and small adult size.

A hypothesis that can be tested experimentally or by comparison is that brood volume increases only as a function of available surface area, while body (and hence, gonad) volume increases as a function of body volume. This would lead to an allometric limitation in available brood space with increasing body size, so that large females could produce more eggs than they could brood (Strathmann and Strathmann, 1982; Strathmann *et al.*, 1984).

Brooding may also place constraints on the embryos or egg coverings themselves. As previously noted and as illustrated in Figure 6, *Lepidochitona* brooders have much more reduced egg hull sculpturing than free spawners. This general pattern holds true when the egg hulls of other chiton brooders are compared to the hulls of their free-spawning congeners (Eernisse, 1984). A hypothesis related to the allometric limitation hypothesis is that a reduction of egg hull sculpturing is under selection to permit tighter packing of eggs within the brood space. Evidence for this hypothesis would provide an indication that brood space is indeed limiting. However, an alternative hypothesis may equally well explain the "smooth" egg hulls of brooders. If the elaborate egg hulls function in some way as planktonic adaptations (*i.e.*, to facilitate suspension via chain formation or to discourage predators) and the hulls are costly to produce, then brooded embryos may be released from such a selective force. Perhaps comparisons with the egg hulls of benthic egg layers, where no brood space constraints or pelagic selective forces should apply, will help distinguish among these alternative hypotheses.

Another allometry hypothesis is that respiration or successful ventilation of a brooded egg mass is increasingly restricted with increasing body size. Such would be the case if gill surface area does not increase at the same rate as gill volume so that, as body size increases, the increase in respiratory efficiency does not keep pace with potentially greater brood output. The placement of brooded chiton embryos within the pallial groove certainly must impede normal respiratory patterns, as Heath (1905) first suggested. Heath (1905) noted but did not specify in detail an observation that brooding *L. caverna* appeared to greatly increase the respiratory powers of their mouth cavities and skin surfaces during brooding. The extent to which chitons can "breathe" through these other body surface areas may also be subject to allometric limitations, with larger animals able to use this means of respiration to a lesser extent. However, this reasoning assumes it is the adult that is respiration-limited. It may be preferable to consider the limitations imposed on the individual embryos of a brood mass. Although these allometric hypotheses are appealing, it is technically difficult to accurately measure either relative volume or respiration in large and small brooding *Lepidochitona*. Again it might be useful to consider chiton species known to lay benthic egg masses which, as discussed above, are like brooders in having late hatching embryos but, because they do not brood, have no increasing respiratory demands associated with increasing body size. The fact that the only two well-documented examples of this habit, *Stenoplax heathiana* Berry, 1946 and *Ischnochiton acomphus* Hull & Risbec, 1930, are also species with large adult size, suggests that such a relationship with body size is plausible.

Life history variation in *Lepidochitona* is considerable yet can be reduced to relatively few factors. The larvae of these morphologically similar brooders or free spawners differ primarily in their potential to remain near their parent(s). Adults differ in their patterns of distribution in the field and, in two out of three species that brood, in their hermaphroditic condition. I have emphasized the potential consequences of low dispersal parental care, while cautioning that some life history traits shared by brooders may be better explained by less obvious morphological or other constraints imposed by brooding itself.

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Development and Larval Morphology of the Spiny Scallop, *Chlamys hastata*

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Abstract. The early life history of *Chlamys hastata,* the spiny scallop, from spawning through metamorphosis to a benthic juvenile is described using light and electron microscopy. Newly released oocytes were about 70 μm in diameter and occasionally were surrounded by a 65 μm-thick jelly coat. A low envelope that is elevated at fertilization was observed in SEM preparations. Gastrulation results from both epiboly and invagination. Primary trochoblasts can be distinguished as two groups of ciliated cells surrounding the blastopore. The D-stage veliger developed by about 50 hours (12°C) and the planktotrophic larval stage is about 40 days in duration. Veliger larvae reached a maximum valve length of 240 μm. Provinculum length remained constant throughout larval life and a ligament may be present in the larval stage. An interlocking crown and groove feature on the larval denticles is described. There is a group of distinctive compound cilia situated at the mouth region that may function in particle sorting. Development of *C. hastata* is superficially similar to that of other species of pectinids. However, differences in details of larval morphology suggest there is greater variation in larval form and function than is generally assumed.

Introduction

Embryonic development and larval morphology of several species of lamellibranch has been described (see review by Wada, 1968; Andrews, 1979; Sastry, 1979; Verdonk *et al.*, 1983). Species that have been examined include *Ostrea edulis* (Waller, 1981; Cranfield, 1973a, 1973b; Hickman and Gruffydd, 1971), *Crassostrea virginica* (Elston, 1980), and *Mytilus edulis* (Lane and Nott, 1975; Bayne, 1971). However, there are only a few studies that provide details on larval organs in pectinids. Cragg and Nott (1977) examined ultrastructure of statocysts in *P. maximus* pediveligers and glands in the foot of *Pecten maximus* were examined by Gruffydd *et al.* (1975). As well, hinge morphology of *Chlamys varia* (LePennec, 1980), *C. distorta, C. opercularis,* and *P. maximus* (LePennec, 1978, from LePennec, 1980) has been described.

Chlamys hastata is found from the Gulf of Alaska to southern California at depths to 150 meters (Bernard, 1983). It is primarily dioecious, reaches sexual maturity at two years, and is thought to spawn annually during the summer months. Adults attain a maximum valve height of 8 cm and generally live about 4.5 years (Grau, 1959, B. MacDonald pers. com.). *C. hastata* supports a small fishery (68.5 tonnes in 1986; R. Harbo, pers. comm.) yet little is known about its early life history.

The objective of this report is to describe and document embryonic and larval development of *C. hastata,* from gamete release through metamorphosis to the benthic juvenile. Morphology of several larval organs, valves, velum, foot, and gill rudiment, is also described.

Materials and Methods

Larval culture

Adult *C. hastata* were collected between June and August by SCUBA diving near Wizard Rock in Barkley Sound, British Columbia, and kept in a darkened, 20 l tank for two to five weeks and supplied with a continuous flow of seawater (14–16°C). Three times daily, several liters of supplemental phytoplankton (2–3 × 10^6 cells/ml) were added.

Scallops were taken from the holding tank for spawning, epifauna removed, and the sexes separated. Seawa-

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ter, irradiated by ultraviolet light, was dripped into the spawning buckets and the water warmed from 12°C to 18°C over a period of one to two hours. Males generally spawned after 20 to 60 minutes and females took up to three hours.

Gametes were collected and immediately washed with 0.8 μm glass-filtered seawater. The oocyte suspension was passed through a 253 μm screen to remove debris, and collected and rinsed on a 20 μm screen. The sperm suspension was passed through a 100 μm screen and added to the oocytes. After 6–10 min, fertilized oocytes were rinsed to remove excess sperm and transferred to 0.45 μm membrane-filtered seawater (FSW) (12°C). Water was changed twice during embryonic development and D-stage veliger larvae were transferred to 2 l vessels containing FSW (16°C) 50 mg/l streptomycin sulphate and 2 mg/l chloramphenicol. Larvae were fed phytoplankton daily (1–4 × 10^5 cells/ml) and water changed every 2–3 days. Phytoplankton used for this study included *Nannochloris atomus, Pavlova lutheri, Isochrysis galbana, Thalassiosira weissflogii,* and *Dunaliella tertiolecta.*

Micrography

Larvae and post-larvae were photographed while swimming or crawling, or while contained within a small piece of Nitex mesh placed on a microscope slide. Occasionally, larvae were narcotized with isotonic $MgCl_2$ to observe larval structures.

For scanning electron microscopy (SEM), valves of larvae and juveniles at different stages were cleaned in distilled water for 30 min to remove soft tissues (Calloway and Turner, 1978) followed by immersing the valves in a 6% solution of sodium hypochlorite for approximately 10–15 min. Cleaned valves were stored in 70% ethanol until required for SEM (Lutz *et al.*, 1982). Valves were disarticulated by shaking vigorously in 6% sodium hypochlorite. Specimens were then rinsed in distilled water and mounted on double-sided sticky tape, sputter coated with gold, and viewed using a Jeol JSM-35 scanning electron microscope. Prior to photographing, each specimen was positioned so that four points along the shell margin, each at 90° intervals, were in exactly the same plane of focus to ensure accurate measurements (Lutz *et al.*, 1982).

Larvae and post-larvae at different stages were relaxed using 1:1 15% solution of $MgCl_2$ and seawater. Specimens were initially fixed for 1 h at room temperature in 2.5% gluteraldehyde, in 0.2 *M* phosphate buffer (Millonig, 1961) and 0.14 *M* NaCl (Cloney and Florey, 1968). Specimens were then rinsed in 0.2 *M* phosphate buffer containing 0.34 *M* NaCl, post-fixed 1 h at 7°C in 2% OsO_4 in 0.2 *M* phosphate buffer, and rinsed twice for 10 min in distilled water. Specimens were dehydrated in a graded series of ethanol and critical point dried using CO_2 as a transitional fluid. Specimens were mounted on stubs and gold coated.

For histological sections, specimens were relaxed and fixed as above. Following secondary fixation the specimens were decalcified in a 1:1 solution of 0.4% ascorbic acid and 0.34 *M* NaCl for 24–48 h (modified from Dietrich and Fontaine, 1975). After being rinsed in distilled water, dehydrated in a graded series of ethanol, and embedded in Epon (Luft, 1961), serial sections were cut, mounted, and stained with 1% azure II and 1% methylene blue in 1% sodium borax solution (Richardson *et al.*, 1960).

Results

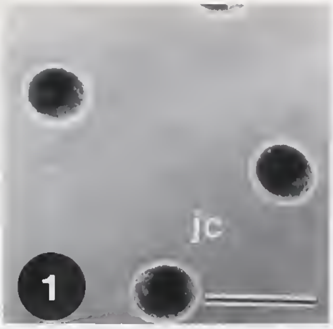
Embryonic development

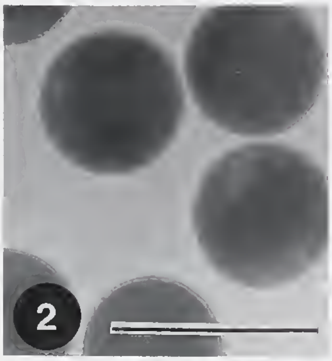
Spawned oocytes are about 70 μm in diameter. On two occasions, a 65-μm thick, translucent jelly coat surrounded each oocyte, making the total diameter about 200 μm (Fig. 1). The jelly coat was not evident using Koehler illumination but could be seen with phase contrast optics. Oocytes from most spawnings did not have a jelly coat (Fig. 2), and fertilization success and subsequent development of oocytes with or without a jelly coat was identical. Germinal vesicles appeared as a translucent region on the periphery of the oocyte.

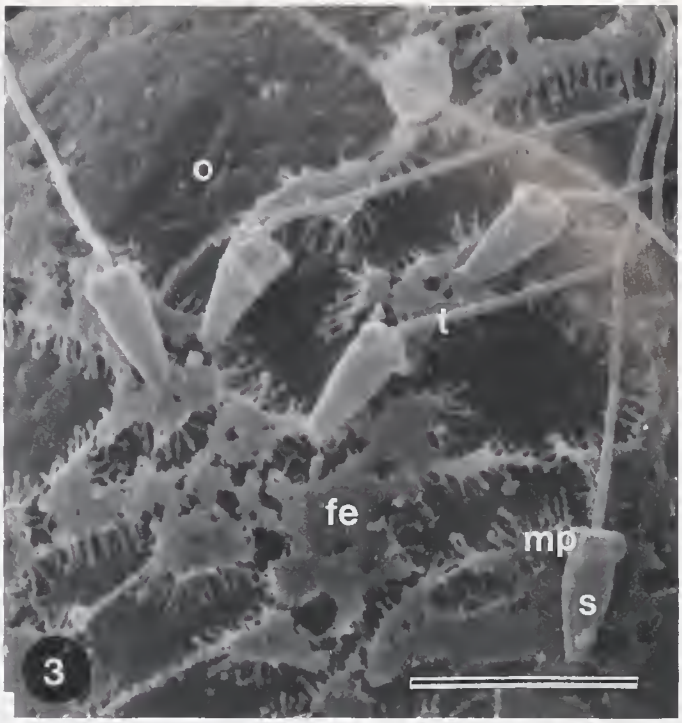
Sperm of *C. hastata* remain active for at least 2 hours after release. Head and midpiece of sperm is about 4 μm long and tail is 40 μm long (Fig. 3). The conical sperm head is 1.25 μm wide at the base and tapers to 1 μm wide at the tip. A 1.25 μm long acrosome is situated at the tip of the sperm.

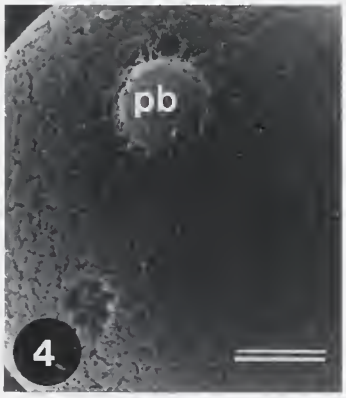
A fertilization envelope and underlying vitelline space are apparent using SEM (Fig. 3). The fertilization envelope is a thin sheet supported by cytoplasmic extensions of the oolemma. The vitelline space is 0.8–1.0 μm thick and the egg surface appears slightly pitted.

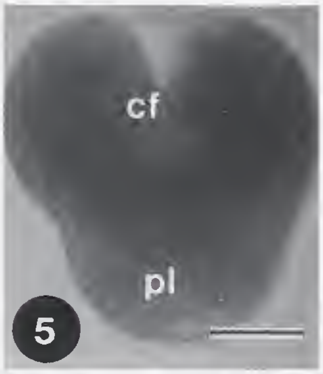
Polar body formation begins about 20 min after fertilization (12°C) (Fig. 4). First cleavage is meridional, unequal, and initiated 3 h after fertilization (Fig. 5). A deep cleavage furrow begins at the animal pole, the site of polar body release, and a large polar lobe forms at the vegetal pole. The polar lobe fuses with the CD blastomere before first cleavage is complete (Figs. 6, 7). Second cleavage is also meridional, perpendicular to the first cleavage plane and is complete by 3.75 h. A, B, and C blastomeres are of equivalent size and the D blastomere is slightly larger. Third cleavage is latitudinal and dexioplectic and unequal (Fig. 8). Within 15 h, the embryo has reached the morula stage, a non-motile ball of cells. Gastrulation occurs by epiboly and invagination is complete by 18 h (Figs. 9, 10). The micromeres extend over

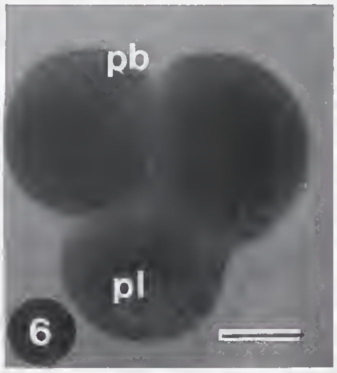


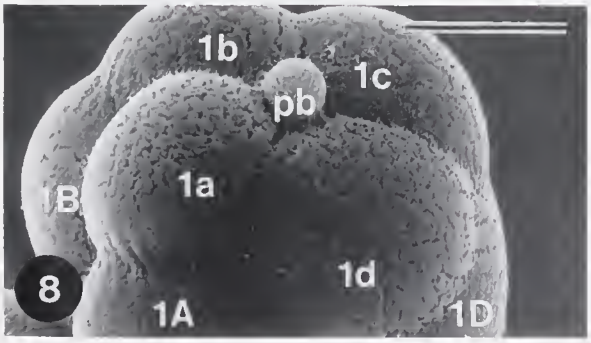












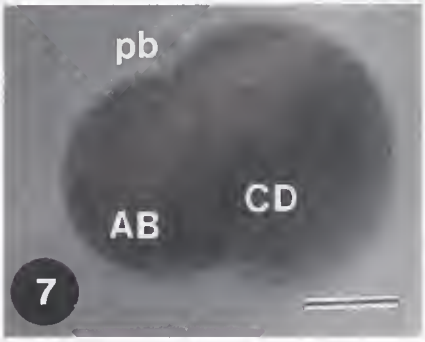


Figure 1. Primary oocytes with jelly coats (jc). Light micrograph (LM), phase contrast optics. Scale bar = 100 μm.

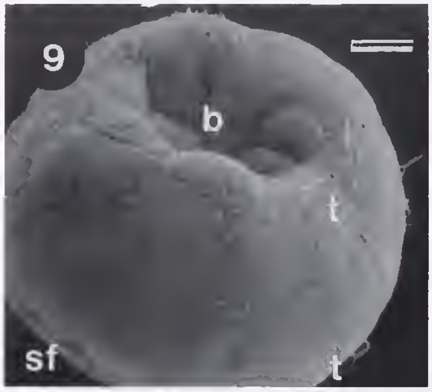
Figure 2. Primary oocytes lacking jelly coats. LM, bright field optics. Scale bar = 100 μm.

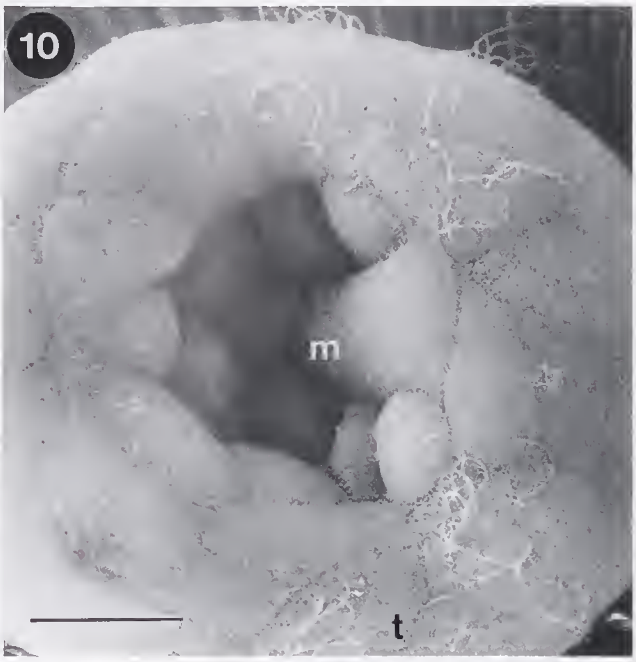
Figure 3. Scanning electron micrograph (SEM) of sperm cells (s) on surface of oocyte at fertilization. mp, midpiece; o, oolemma; t, tail; fe, fertilization envelope. Scale bar = 5 μm.

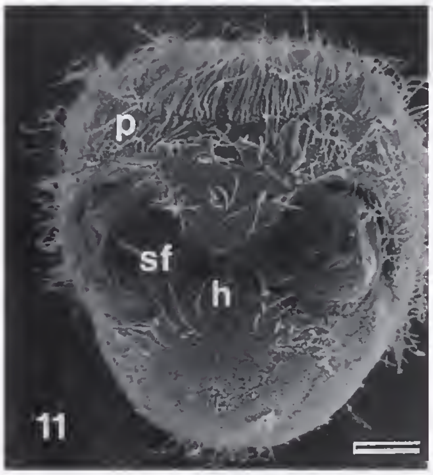
Figure 4. SEM of first polar body (pb) release. Scale bar = 10 μm.

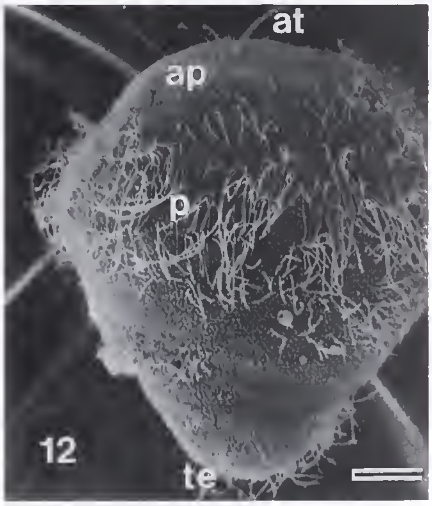
Figures 5–7. First cleavage with polar lobe formation. Polar lobe (pl) forms at vegetal pole as cleavage furrow (cf) develops at animal pole. Polar lobe fuses with CD blastomere as cleavage becomes complete. LM, bright field optics. pb, polar body. Scale bar = 20 μm.

Figure 8. Third cleavage stage. Blastomeres labelled using nomenclature for spirally cleaving embryos. SEM. pb, polar body. Scale bar = 20 μm.









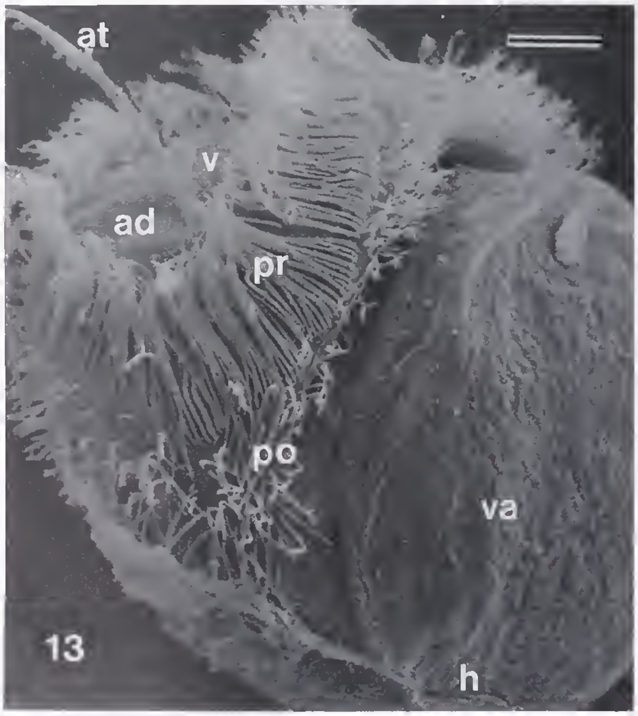


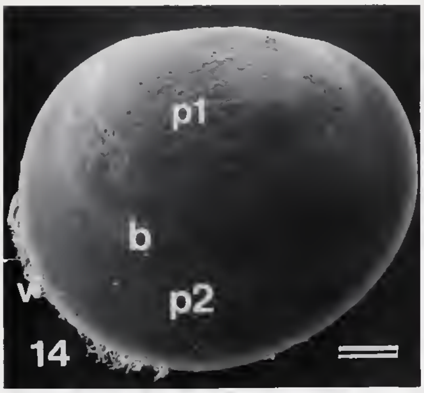
Figure 9. Blastopore (b) is initially a wide, shallow depression at vegetal pole. Ectodermal cells invaginate to form shell field (sf) on dorso-lateral surface. Two groups of ciliated cells, the primary trochoblasts, (t) are evident on perimeter of blastopore and on dorso-lateral surface. SEM.

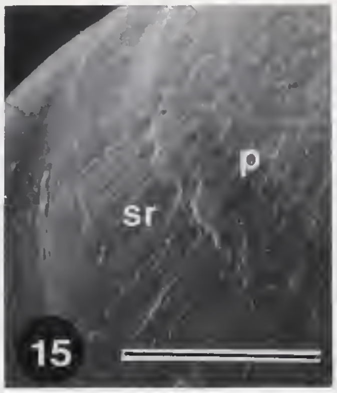
Figure 10. Ventral view of blastopore. Invagination is deeper and perimeter of margin is narrower than in Figure 9. A single macromere (m) can be seen in the blastopore. SEM. t, primary trochoblasts.

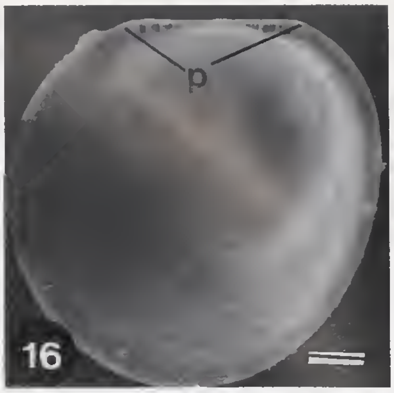
Figure 11. Trochophore larva with developing shell field (sf) on dorsal surface. Shell secretion is initiated in two regions. SEM. h, future hinge; p, prototroch.

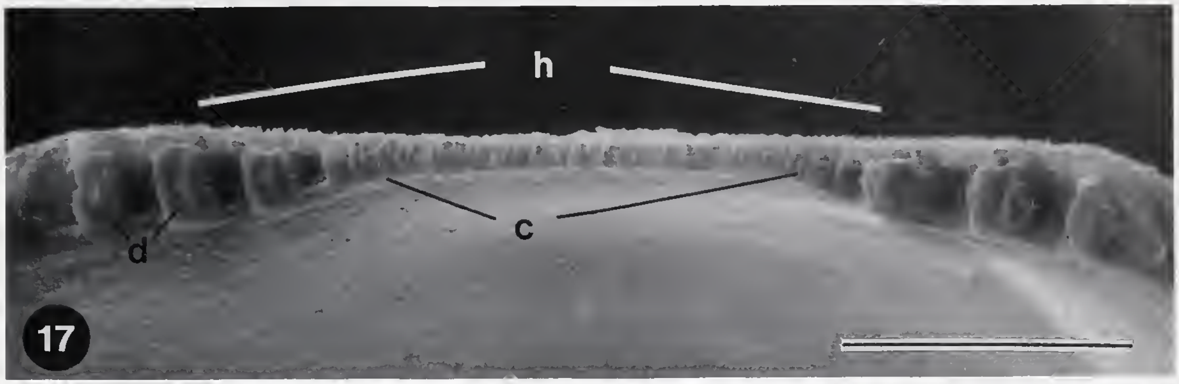
Figure 12. Trochophore larva. SEM. at, apical tuft; ap, apical plate; p, prototroch; te, telotroch.

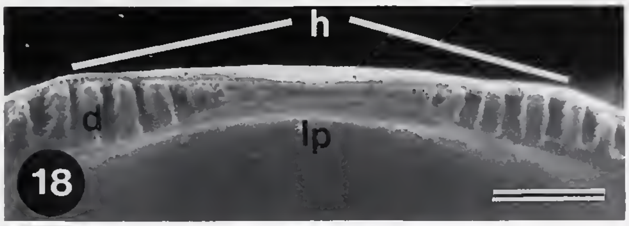
Figure 13. Early D-stage larva, 45 h after fertilization, anterior view. Velum has two ciliated bands on outer margin. SEM. ad, apical disc; at, apical tuft; h, hinge region; pr, preoral band; po, postoral band; v, velum; va, valve. All scale bars = 10 μm.

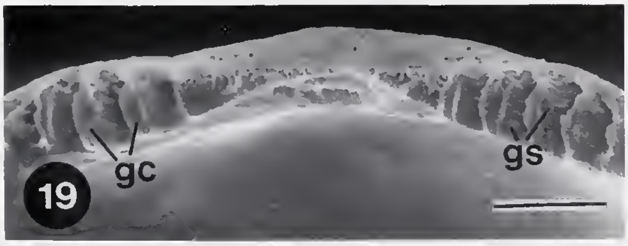












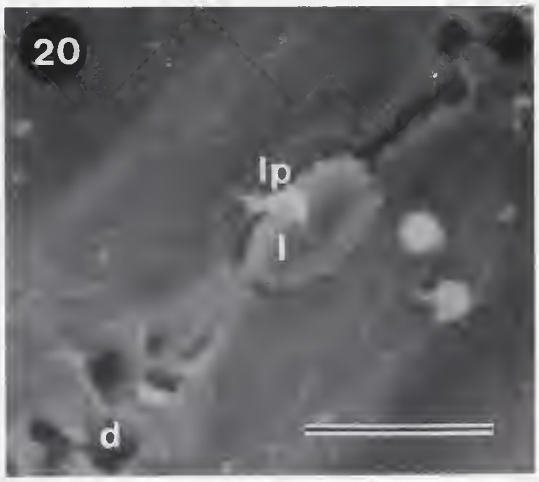


Figure 14. SEM of external surface of left valve of 15-day larva. b, prodissoconch I/II boundary; p1, prodissoconch I shell; p2, prodissoconch II shell; v, velar cilia.

Figure 15. Detail of Figure 14. SEM. p, punctate region and sr, stellate-radial region of prodissoconch I shell.

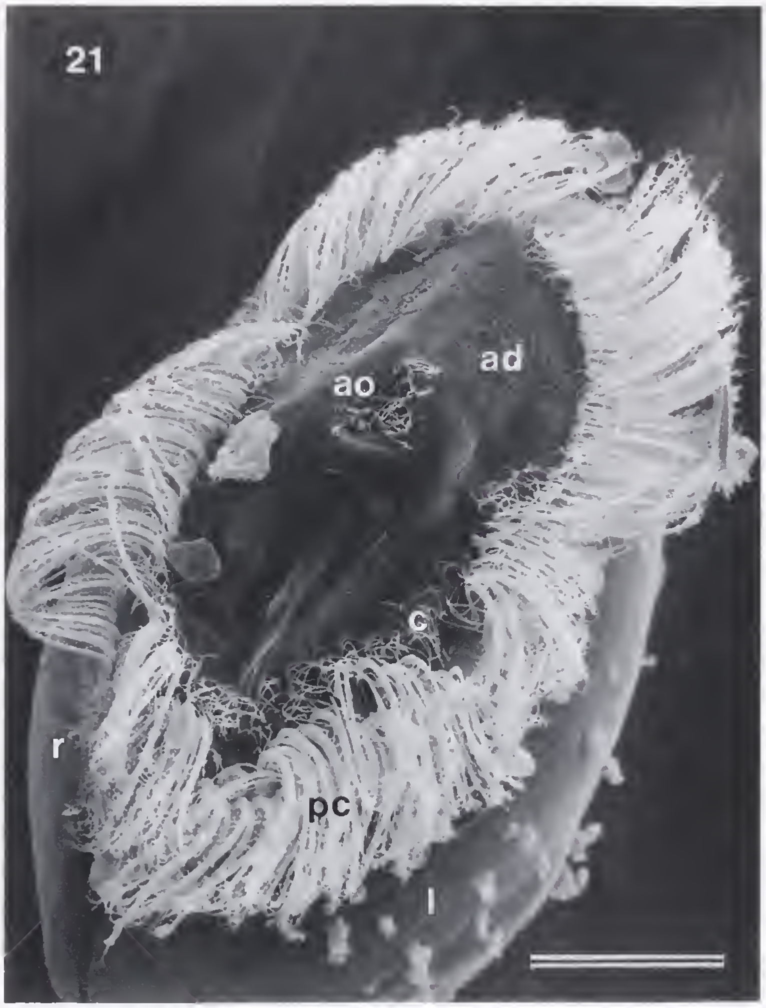
Figure 16. Internal surface of left valve of 15-day larva. SEM. p, provinculum.

Figure 17. Detail of Figure 16. SEM. d, denticles; c, cardinal region; h, hinge line.

Figure 18. Provinculum of 39-day larva. SEM. d, denticles; h, hinge line; lp, ligament pit.

Figure 19. Provinculum of 39-day larva. Note the crown of each denticle bears a groove (gc) and the sides bear transverse grooves (gs). SEM.

Figure 20. Hinge region of 39-day larva. SEM. d, denticles; l, ligament; lp, ligament pit. All scale bars = 20 μm.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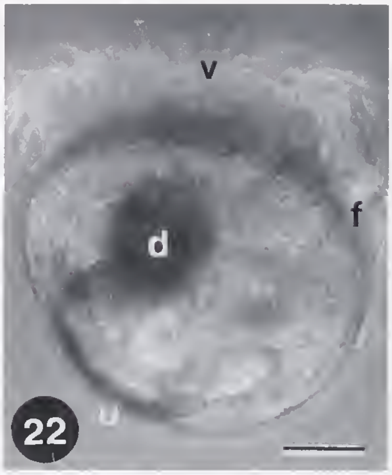
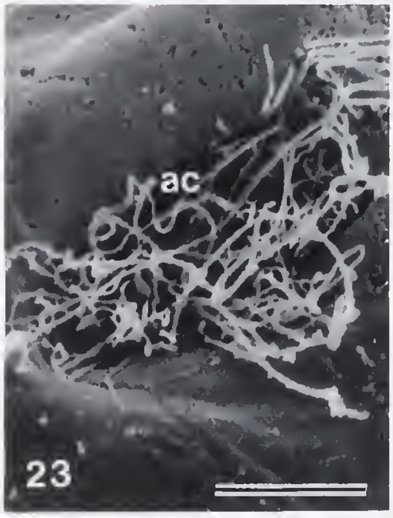
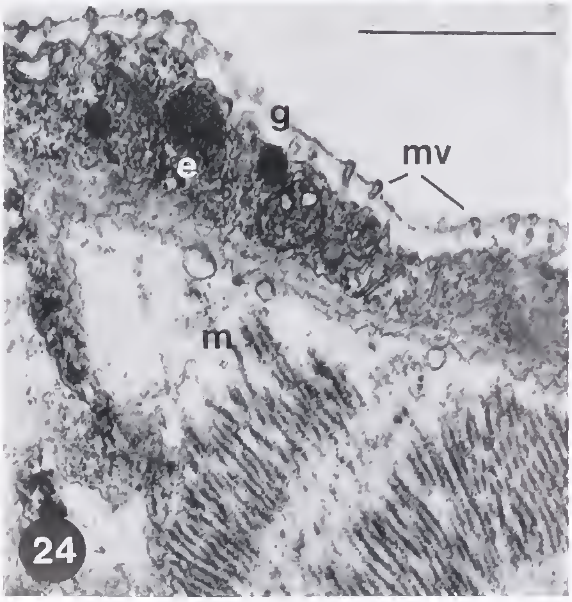






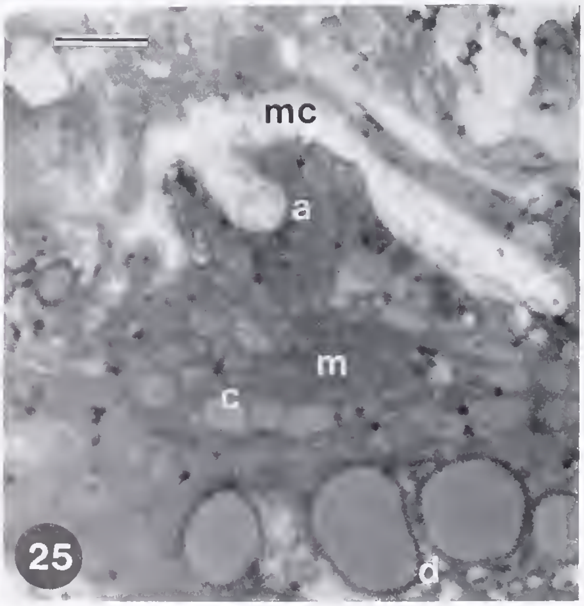


Figure 21. SEM of pediveliger larva with opened velum. A sparse band of simple cilia (c) is situated between the preoral cilia (pc) and the apical disc (ad). ao, apical organ; l, left valve; r, right valve; Scale bar = 50 μm.

and enclose the macromeres and then appear to sink into the blastopore. The blastopore is wide and shallow initially, but the opening becomes smaller as the invagination deepens. On the perimeter of the blastopore are two groups of 2–4 cells bearing sparse cilia, the primary trochoblasts (Fig. 10). Two other groups of primary trochoblasts girdle the embryo between the animal and vegetal poles (Fig. 9).

At 21 h, gastrulae hatch from their fertilization envelope and by 30 h trochophores swim near the surface of the water (Fig. 11, 12). The prototroch develops from the primary trochoblasts and encircles the mid-region of the trochophore as a 24 μm broad band of widely spaced, simple cilia. The apical tuft, a group of 8–10 fused cilia, is situated centrally on the apical plate, and projects forward 10–15 μm (Fig. 12). The posterior apex of the trochophore bears a small tuft of cilia, the telotroch, and the rest of the body wall is sparsely ciliated.

The shell field, located on the dorsal surface of the trochophore, begins secretion of the prodissoconch I shell in two regions (Fig. 9, 11). These two regions contact along the straight hinge line. Using Koehler illumination, larval valves are first apparent in 45-h-old larvae, but are not capable of closing completely (Fig. 13). Using cross polarized filters, birefringence was observed, indicating that the valves are calcified at this time. By 50 h the embryo has developed into a D-stage veliger. The valves are capable of closing and surrounding the entire larva. At this stage valve length is about 105 μm and valve height is 82 μm. The prototroch transforms into the velum of the veliger larva with two distinct rows of cilia, a row of 20-μm long compound cilia, the preoral band, and a row of shorter, simple cilia, the postoral band.

Larval morphology

Valves. The shell gland of the trochophore becomes the mantle of the D-stage larva and continues secretion of the valves. Two distinct types of shell are secreted, the prodissoconch I and prodissoconch II (Fig. 14). The prodissoconch I is comprised of two distinct regions, an area uniformly dimpled with shallow pits and a zone about 12–14 μm broad which is convex and radially striate (Fig. 14). Carriker and Palmer (1979) referred to this pattern as the punctate-stellate pattern. The prodissoconch II is smoother and is commarginally striate (after Waller, 1981).

The hinge region, or provinculum, of 15-day-old larvae consists of a narrow, smooth ridge along the hinge line with 3–4 small stout denticles at either end in a narrow, triangular depression (Fig. 16, 17). Development of the denticles at both ends is symmetrical. Mean hinge length in 15-day larvae is 75.3 ± 15.6 μm, and mean provinculum length is 100.5 ± 21.8 μm. (n = 4). In pediveligers, the ridge along the hinge line is thicker and five to six denticles are present at either end (Fig. 18). Provinculum length, or distance between outer margins of outside denticles, remains constant throughout larval development, whereas the distance between the inner margins of the inside denticles is less in 39-day-old larvae (28.1 ± 9.9 μm (n = 2) *versus* 41.3 ± 12.7 μm (n = 4) for 15 day larvae). This indicates that additional denticles are added to the inside of the initial three denticles. In pediveligers, denticles are more pronounced and slightly longer than those of younger larvae and have a columnar appearance. The crest of each denticle is slightly indented along the long axis such that there are two small ridges perpendicular to the hinge (Fig. 19). The anterior and posterior sides of the denticles bear curved ridges which are concave toward the hinge line.

A ligament pit is evident in larvae about 210 μm in valve length as a shallow depression immediately below the hinge line in the center (Fig. 18, 19). The ligament is broad (about 15 μm) and takes up almost one-half of the area between the denticles (Fig. 20).

Maximum valve length in *C. hastata* pediveligers is 240 μm (39 days after fertilization). Larvae held longer than 40 days did not show an increase in valve length or height.

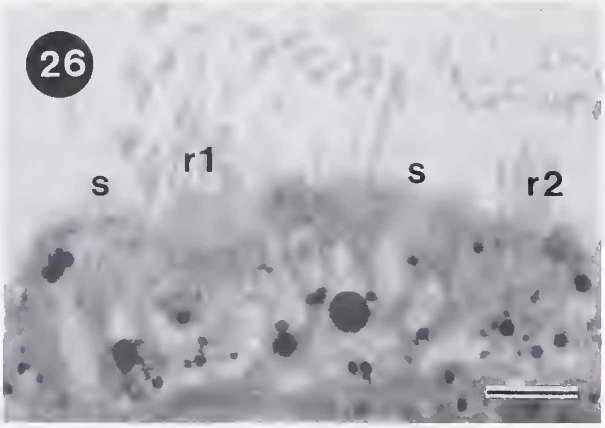
Velum. The velum is the oval locomotory and feeding organ of the larva (Figs. 21, 22). When extended, the ciliated margins of the velum protrude beyond the anterior edges of the valves. When retracted, the velum is folded anterior-posteriorly and occupies the anterior half of the mantle cavity. Retraction is achieved by the striated anterior and posterior retractor muscles, which have sev-

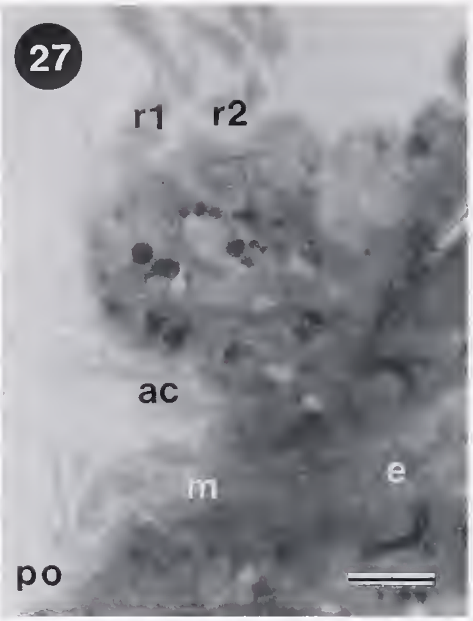
Figure 22. Swimming pediveliger larva with foot (f) extended. LM, phase contrast optics. d, right digestive diverticulum; u, umbo; v, velum. Scale bar = 50 μm.

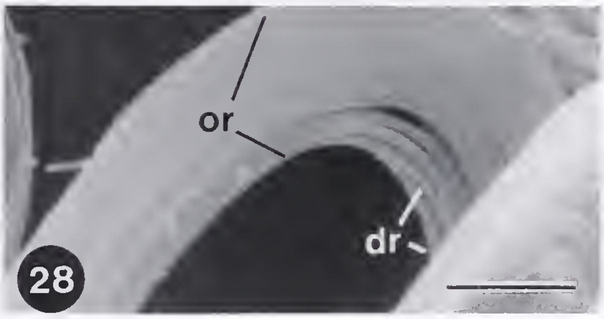
Figure 23. Detail of Figure 21. Note apical cilia (ac) emerging from apical organ. SEM. Scale bar = 10 μm.

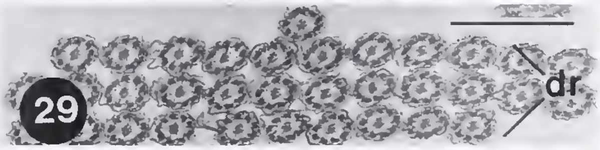
Figure 24. TEM of velar disc epithelium. e, epithelial cell; g, glycocalyx; m, muscle cell; mv, microvilli. Scale bar = 10 μm.

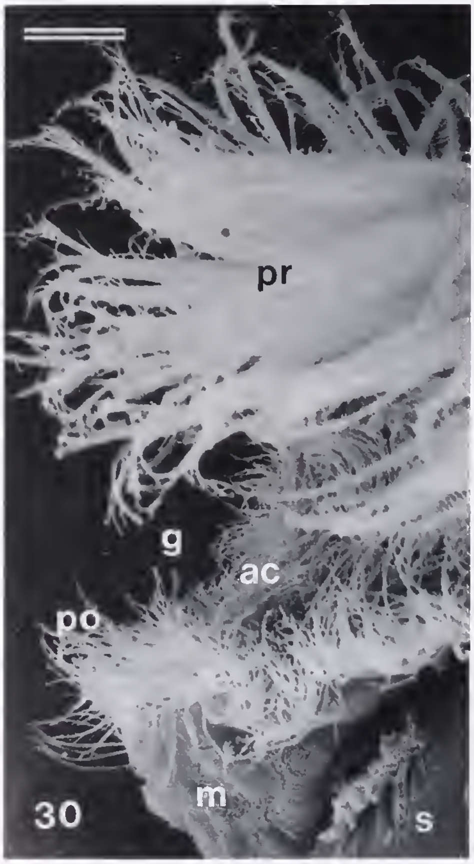
Figure 25. Histological section of apical organ and cerebral ganglion in pediveliger larva with partially retracted velum. a, apical cells; c, cortex and m, medula of cerebral ganglion; d, digestive diverticulum; mc, mantle cavity. Scale bar = 10 μm.











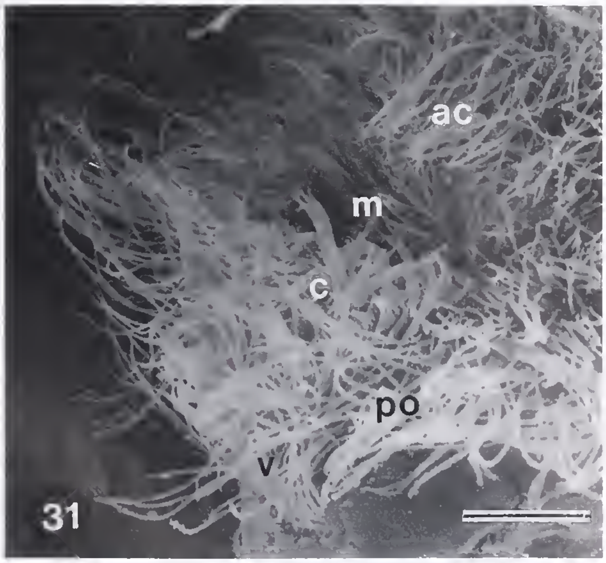


Figure 26. Tangential histological section of preoral band of velar margin. The preoral band consists of two rows of compound cilia (r1, r2) with secretory cells (s) situated between two rows and between the preoral and adoral ciliary bands. Scale bar = 10 μm.

Figure 27. Histological cross-section through ciliated bands on velar margin at the mouth region (m). ac, adoral cilia; e, esophagus; po, postoral cilia; r1, r2, double row of compound cilia of preoral band. Scale bar = 10 μm.

Figure 28. SEM of compound cilia of preoral band showing long orthoplectic rows (or) and short diaplectic rows (dr). Scale bar = 2 μm.

eral insertions on the epithelium of the velum along the anterior-posterior axis.

The apical plate of the trochophore remains as the central portion of the velum or apical disc. The apical tuft of the trochophore persists for 2–3 days in D-stage veligers. In older larvae this region becomes the apical pit, a small, deep invagination located in the center of the apical disc. The apical pit is oval in shape, approximately 6–8 μm wide, 20 μm long, and 10 μm deep (Fig. 23). Numerous fine, simple cilia originate from the apical pit and project a short distance beyond the rim of the pit. The epithelium of the apical disc is comprised of simple squamous to cuboidal cells that have a thick glycocalyx and numerous microvilli on their apical surfaces (Fig. 24). Cells of the apical pit are columnar, approximately 7 μm high, with apical nuclei. Directly underlying these cells is the cerebral ganglion (Fig. 25).

On the outer margin of the velum of D-stage veligers, compound cilia of the preoral band are arranged in a single row, and each compound cilium consists of two or three cilia adherent throughout their length (Fig. 13). In older larvae the preoral band consists of two rows of compound cilia (Figs. 26, 27). Each compound cilium is arranged in 6–15 orthoplectic rows and 2–6 diaplectic rows of cilia (Fig. 28, 29). Cilia comprising each compound cilium fit closely together along most of their length (Fig. 30). Cilia of the postoral band are simple and short (8 μm) and are arranged in one or two closely spaced rows (Figs. 27, 30). A third band of cilia between these two bands, the adoral band, is approximately 24 μm wide and the cilia are 8 μm long (Figs. 27, 30). A fourth, poorly defined band of 17 μm long simple cilia is situated between the apical plate and the preoral band (Fig. 21).

On the ventral surface of the velum the postoral band extends posteriorly and forms a V-shaped ventral lip, called the postoral tuft by Waller (1981) (Fig. 31). The mouth is situated anterior to the ventral lip, between the postoral and adoral cilia. Several short, broad compound cilia are located immediately ventral to the mouth. Each compound cilium consists of cilia not less than 8 μm in length arranged in a row of seven to nine cilia. The compound cilia are interspersed among the simple cilia of the postoral band.

In cross section, the outer margin of the velum is bilobed with the preoral and postoral bands at the apex of either lobe and the adoral band in a shallow trough between them (Figs. 27, 30). Cells of the ciliated bands are more columnar than other epithelial cells (Figs. 26, 27). Cells of the preoral band have large vacuoles in the basal region of each cell. Between the two rows of preoral cilia, and between the preoral and adoral bands, there are several secretory cells with large granules in their apical region (Figs. 26, 27).

Foot. The foot is first apparent in 15-day-old larvae as a small ciliated rudiment in the posterior-ventral region between the mouth and anus. This rudiment, the prepodium, forms the metapodium of the foot. About 28 days after fertilization the propodium develops rapidly from the anterior portion of the prepodium. By 34 days the foot is functional and larvae are often observed crawling on the substrate or swimming with the foot extended (Fig. 22). The foot is bilaterally symmetrical, about 110 μm long and 53 μm wide with a distinct toe and heel (Fig. 32). Lateral and dorsal surfaces of the foot are sparsely ciliated (Fig. 33). The ventral surface is covered with long (8–24 μm), simple cilia (Fig. 32) and has a byssal groove, 48 μm long, extending along the midline of the longitudinal axis. At the posterior-most region of the heel is a tuft of cilia (Fig. 32).

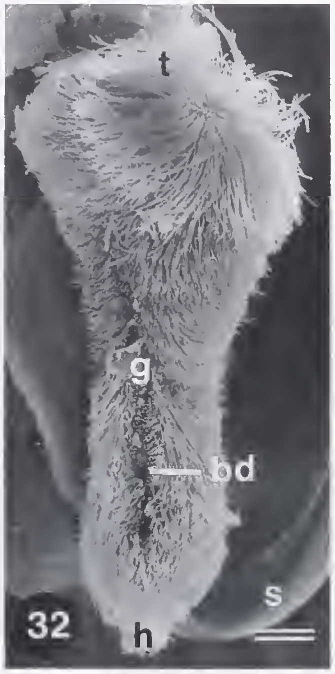
The primary byssus gland stains dark blue with Richardson's stain and is situated in the posterio-dorsal region of the foot, immediately posterior to the pedal ganglion (Fig. 34). Ventral to the primary byssus gland is an open, ciliated region—the lateral pouch—with two byssal ducts that pass ventrally to the sole of the foot (Fig. 34). Each duct is densely ciliated and lined with several secretory cells which probably also pass secretion granules into the duct. The ducts open at the posterior region of the byssal groove on the sole of the foot (Fig. 32).

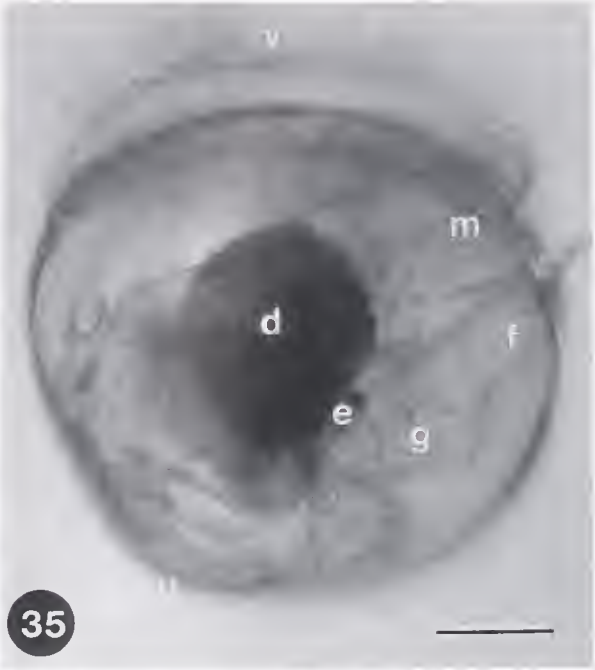
When crawling, both ciliary and muscular action aid in forward movement of the foot along the substrate. The remainder of the larval body is dragged along the substrate, behind the foot, with the hinge region posteriormost; the body is periodically moved forward by sharp contraction of the pedal retractor muscles. The tip of the foot occasionally lifts off the substrate or moves laterally in a swaying motion during forward movement, giving the impression of a sensory function. The velum is extended while crawling and probably aids in the forward movement of the larva. Periodically, the larva will lift off

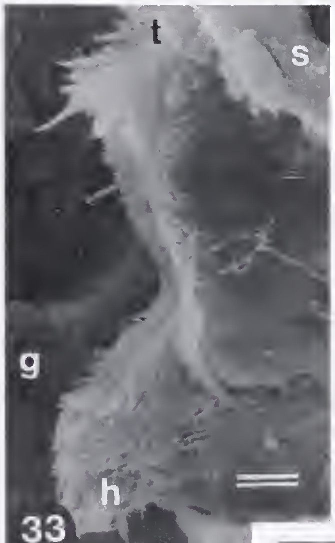
Figure 29. TEM of cross-section of a single compound cilium of preoral band showing arrangement of cilia within a compound cilium. dr, diaplectic row. Scale bar = 0.5 μm.

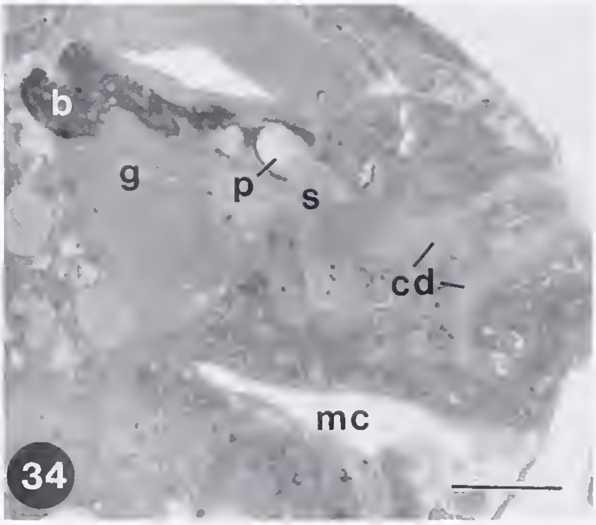
Figure 30. Cross-sectional view of ciliated bands on velar margin. SEM. ac, adoral cilia; g, food groove; m, mantle tissue; pr, preoral cilia; po, postoral cilia; s, shell. Scale bar = 10 μm.

Figure 31. Mouth region at posterior edge of velar margin. The mouth (m) is situated between the adoral (ac) and postoral (po) ciliary bands. Several short, compound cilia (c) are located just ventral to mouth. SEM. v, ventral lip. Scale bar = 10 μm.





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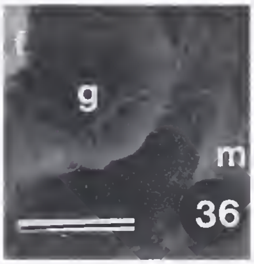
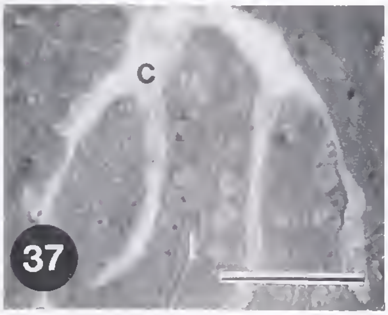




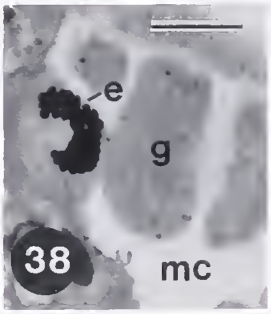


Figure 32. SEM of ventral surface of foot of pediveliger larva. Note dense ciliation on sole of foot with byssal groove (g) running medially from toe (t) to heel (h). SEM. bd, region of byssal duct; s, shell. Scale bar = 10 μm.

Figure 33. Lateral view of foot of pediveliger larva. Note dense cilia on sole of foot and only sparse cilia on lateral surface. SEM. g, gill rudiment; h, heel; s, shell; t, toe. Scale bar = 10 μm.

the substrate and swim, leaving the foot extended (Fig. 22).

Gill rudiment. Gill rudiments are first visible in larvae about 26 days old. On either side of the foot, a ridge of tissue—the gill plate—extends from the mantle into the mantle cavity. From each gill plate three small lobes of tissue, the primary gill filaments, develop (Fig. 35). By 32 days each rudiment is about 22 μm long and is sparsely ciliated with a single row of simple cilia along the apical margin that beat inward with an anteriorly directed metachronal wave (Fig. 36).

The primary filaments consist of a simple cuboidal epithelium with large vacuoles filled with granules (Fig. 37). Each filament has a narrow ciliated lumen. No cellular or ciliary connection between gill filaments on opposing sides of the mantle cavity was observed.

The larval eyes are first visible in larvae about 24 days old (Fig. 35) and are located on the anterior aspect of each gill bar. The larval eye consists of two cells, one with darkly staining granules arranged in a cup shape oriented anteriorly, and the other situated within the cup shape (Fig. 38).

Post-larval morphology

Length of larval development is about 40 days. At the pediveliger stage, valve length is about 240 μm; the foot is functional, and the gill rudiments are distinct. At metamorphosis, *C. hastata* transforms from a swimming pediveliger to a crawling, benthic post-larva within 24–48 hours. The internal organs of the larva undergo a 90° counter-clockwise rotation. The velum is histolized and the mouth moves from a ventral to an anteriodorsal position. As well, the foot moves anteriorly. Approximately 24 hours after metamorphosis has been initiated the foot is in a ventral position (Fig. 43), and by 48 hours after metamorphosis the foot is in an anterior position and individuals can crawl on the substrate with the hinge pointing in the direction of movement. The gill rudiments undergo rapid histogenesis to develop into the adult gills. With the forward rotation of the mouth region and foot, the gill filaments come to occupy the posterior region of the mantle cavity.

Once metamorphosis is complete, the mantle folds begin secretion of the dissoconch shell. Secretion of the left valve is initiated first and is visible in juveniles two days after metamorphosis (Fig. 39). The left valve has strong radial striations and formation of the anterior auricle is evident in early juveniles (Fig. 40). The right valve is smooth and a byssal notch is evident by three or four days after metamorphosis (Fig. 41). The dissoconch shell is secreted with little convexity and growth proceeds more rapidly in the direction of shell height than shell length.

The provinculum is thicker and the ligament pit is larger (19 ± 1.8 μm, n = 6) and more pronounced in 14 day old juveniles (Fig. 42). No further denticles are added after metamorphosis and larval denticles are gradually lost by overgrowth of juvenile shell. A ligament scar is evident at the region of the ligament pit, providing an increased surface to which the ligament attaches (Fig. 42).

At metamorphosis, the primary gill filaments lengthen and more are added (Fig. 43). By 2–3 days after metamorphosis, the single row of simple cilia present in the larval gill rudiments becomes the lateral ciliated band of the adult gill lamellae (Fig. 44). Length of gill lamellae is about 28 μm at this time. Another band of cilia, the frontal cilia, is just beginning to develop by 2–3 days and is clearly visible in 12 day old juveniles (Fig. 45).

Discussion

A jelly coat surrounding newly released oocytes has not been reported in pectinid species other than *C. hastata,* however, jelly coats do surround eggs of *Patinopecten yessoensis* and *Crassadoma gigantea* (CAH, unpub. obs.). The presence of the jelly coat may be common among pectinid species but since it is translucent and difficult to observe using Koehler illumination, it is possible that it has not been noticed by other authors.

Evidence from this study suggests that gastrulation oc-

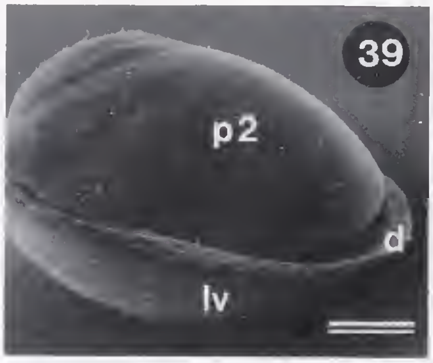
Figure 34. Histological section of foot region. Secretions (s) of primary byssus gland (b) are deposited into the lateral pouch (p) which leads into two ciliated ducts (cd). g, pedal ganglion; mc, mantle cavity. Scale bar = 10 μm.

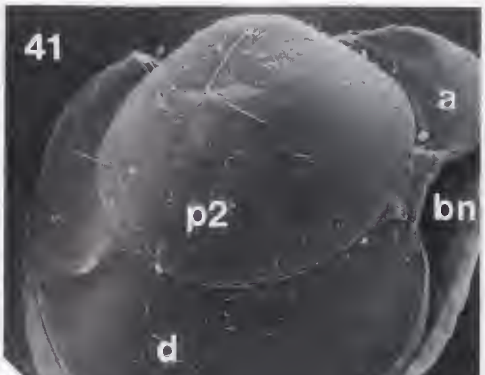
Figure 35. Light micrograph of swimming pediveliger, 40 days after fertilization, with toe of foot (f) extended. LM, bright field optics. e, eyespot; d, right digestive diverticulum; g, right gill rudiment; m, mouth; u, umbo; v, velum. Scale bar = 50 μm.

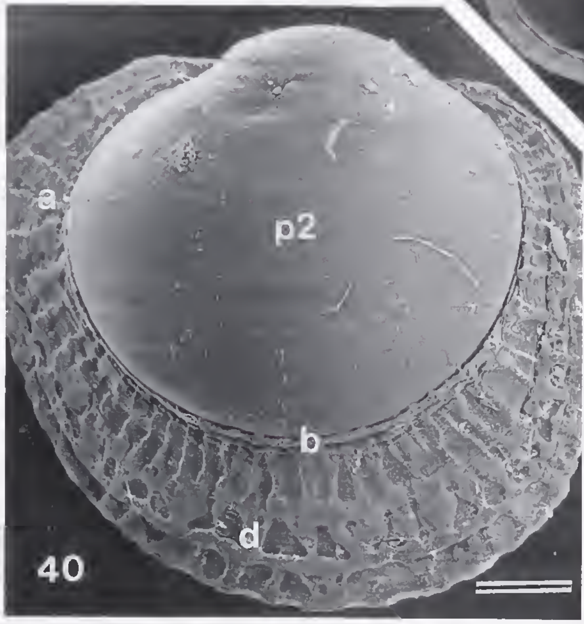
Figure 36. SEM of anterior-most primary gill filament (g) from right gill plate of a pediveliger larva. Note the single row of cilia. f, foot; m, mantle. Scale bar = 10 μm.

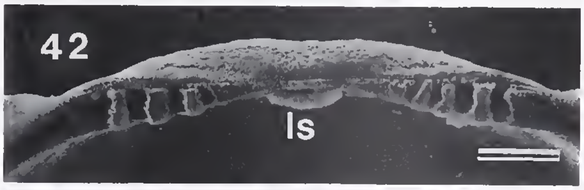
Figure 37. Longitudinal histological section through primary gill filaments. c, cilia; l, lumen. Scale bar = 10 μm.

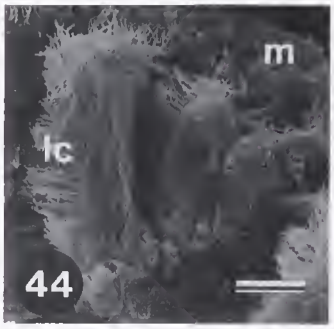
Figure 38. Histological section through primary gill filaments (g) and eyespot (e) of 33-day-old larva. Dense, pigmented granules are deposited in the shape of a cup within a single cell. mc, mantle cavity. Scale bar = 10 μm.











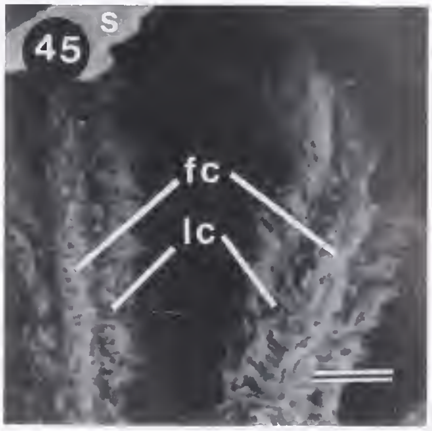


Figure 39. External view of postlarva, two days after metamorphosis, viewed from posterior region. Note dissoconch shell (d) on left valve (lv). SEM. p2, prodissoconch shell. Scale bar = 50 μm.

curs by both epiboly and invagination (Figs. 9, 10). This agrees with observations by Raven (1958) of *O. edulis* embryos. Other lamellibranchs in which this form of gastrulation has been observed include *Ensis, M. edulis* and *Teredo* (Verdonk and Biggelaar, 1983).

The two groups of cells located near the perimeter of the blastopore are probably two groups of the quartet of ciliated cells called the primary trochoblasts. Ultimately, they form the prototroch of the trochophore and ciliated bands of the velum in the veliger stage. These are generally described as originating from four points around the equatorial region of the embryo whereas the blastopore invaginates at the vegetal pole. However, the blastopore is displaced anteriorly during development and the pretrochal region is shifted forward, owing to considerable growth of the dorsal region, and the main axis of the embryo becomes bent such that the blastopore becomes situated in front of the prototroch (Verdonk and Biggelaar, 1983). Since the mouth is situated just below the prototroch, and ciliated bands of the velum, the location of the primary trochoblasts near the blastopore suggests that the blastopore becomes the future mouth.

The prodissoconch I shell of *C. hastata* bears the punctate-stellate pattern as first described by Ansell (1961). In published micrographs of *C. virginica* (Carriker and Palmer, 1979), the punctate region is about 30 μm in diameter, and in *O. edulis* (Waller, 1981) the region is ovoid, about 30 × 50 μm. Carriker and Palmer (1979) suggested that this region probably overlies the embryonic shell gland. However, in *C. hastata* the punctate pattern covers an area of about 40 × 80 μm (Fig. 14), about twice the size of the region of *O. edulis* or *C. virginica*. If indeed the punctate region represented the area of the embryonic shell gland, then the shell gland of *C. hastata* should be twice the size of shell glands of *O. edulis* and *C. virginica*.

The stellate-radial zone presumably represents the region where shell secretion is taken over by the mantle folds (Carriker and Palmer, 1979; Waller, 1981). Waller (1981) suggested that the transition from shell gland secretion to mantle secretion was gradual and occurred long before the prodissoconch I/II boundary in *O. edulis*. In his micrographs, it is apparent that the stellate-radial zone ends long before the prodissoconch I/II boundary and therefore his argument for the prodissoconch I/II boundary simply representing the first time of closure deserves consideration. The stellate-radial zone ends before the prodissoconch I/II boundary in *C. hastata* supporting Waller's hypothesis.

Other pectinids examined for larval hinge morphology are *P. maximus, C. varia, C. distorta* and *C. opercularis* (LePennec, 1980). Shell shape and hinge morphology of *C. hastata* larvae is similar to these species. All pectinids have hinge teeth which are symmetrical at each end with a thin cardinal ridge between which lacks cardinal teeth. *C. hastata* may have up to five or six denticles at each end by the time of metamorphosis whereas LePennec (1980) noted only three denticles in other pectinids. No further denticles were added after metamorphosis in *C. hastata*, which is similar to observations in other pectinids, except in *C. opercularis* which add one or two more denticles after metamorphosis.

In *C. hastata* larvae provinculum length remains constant throughout larval life. This contradicts the findings of Lutz and Hidu (1979) who examined provinculum lengths of *M. edulis* and *Modiolus modiolus* valves. They found that provinculum length increased with valve length and valve height by a linear relationship. The characteristic of a constant provinculum length, combined with hinge structure and valve dimensions, may be of assistance in distinguishing *C. hastata* larvae from other closely related species.

A common characteristic of bivalve larval denticles is the transverse ridges on the sides. *O. edulis* (Waller, 1981), *C. virginica* (Carriker and Palmer, 1979), *C. gigas* (Waller, 1981), *M. edulis* and *M. modiolus* (Lutz and Hidu, 1979), as well as *C. hastata* have transverse ridges. Lutz and Hidu (1979) suggested that the ridges reduce shear between the valves. A groove on the crown of each denticle of *C. hastata* corresponds to a small rise or bump located between each denticle of the opposing valve. No mention of these structures has been made in

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Figure 40. External view of left valve of 12 day old juvenile. SEM. a, anterior auricle; d, dissoconch shell; b, prodissoconch II/dissoconch boundary; p2, prodissoconch II shell. Scale bar = 50 μm.

Figure 41. External view of right valve of 12 day old juvenile. Right valve is overlapped by larger left valve. Note distinct byssal notch (bn). SEM. a, anterior auricle; d, dissoconch shell; p2, prodissoconch II shell. Scale bar = 50 μm.

Figure 42. Proviniculum of 12 day old juvenile. SEM. ls, ligament scar. Scale bar = 20 μm.

Figure 43. Light micrograph of postlarva, one day after metamorphosis. Foot (f) is oriented ventrally and is very flexible (toe of foot is actually pointing posteriorly). Gill lamellae (g) occupy the posterior half of mantle cavity. LM, phase contrast optics. e, eyespot; u, umbo. Scale bar = 50 μm.

Figure 44. Proximal view of left gill lamellae of 4 day old juvenile. Note dense row of lateral cilia (lc) on 2 of the gill filaments. SEM. m, mantle tissue. Scale bar = 10 μm.

Figure 45. Distal view of gill lamellae of 12 day old juvenile. SEM. fc, frontal cilia; lc, lateral cilia; s, shell. Scale bar = 10 μm.

previous literature, and of the published scanning electron micrographs of larval valves in the literature, only *M. edulis* (Lutz and Hidu, 1979; see plate II, Figure A) also has a groove on the crown on each denticle. This crown and groove feature may further assist in the interlocking nature between opposing valves.

Neither the larval ligament nor the ligament pit was observed in other larval pectinids investigated (LePennec, 1980). Lutz and Hidu (1979) suggested the ligament is a post-larval feature, observed only after metamorphosis has been initiated. Larval valves of *C. hastata* capable of metamorphosing (39 days old at 16°C) had a ligament pit. The ligament pit may appear among larvae which are ready to metamorphose and not only in post-larvae.

Dissoconch shell of *C. hastata* is visibly different from, and is secreted at a faster rate than, prodissoconch II shell. These differences probably reflect the differences in the composition of the two types of shell. Prodissoconch I shell is entirely aragonitic (Stenzel, 1964) whereas dissoconch shell is entirely calcitic (Taylor *et al.*, 1969).

Among *C. hastata* post-larvae, the left valve is always slightly larger than the right valve. Even 12 days after metamorphosis, the right valve is about 12 μm smaller than the left valve. Secretion of the dissoconch left valve is initiated before secretion of the right valve and this may lead to the difference in size of the valves. This overlap of the left valve over the right valve has not been reported for other lamellibranch post-larvae.

The bilobe configuration of the outer margin of the velum was first noted by Elston (1980) in *C. virginica* larvae. Bivalve larvae probably feed by the opposed ciliated band method (Strathmann *et al.*, 1972; Strathmann and Leise, 1979) and this configuration may help keep particles in the food groove. According to the opposed ciliated band theory, the adoral cilia are responsible for transporting collected particles toward the mouth region, and the trough shape of the velar margin may assist in retaining particles in the region of the adoral cilia by creating a sunken channel in which the particles travel.

Secretory cells among the velar cilia have not been described previously, although other authors have either observed or assumed that mucus is involved in collection of particles (Yonge, 1926; Erdmann, 1935; Strathmann *et al.*, 1972; Waller, 1981). The mechanism by which veliger larvae are believed to capture food particles is direct interception of particles by the preoral cilia (Rubenstein and Koehl, 1977; Strathmann and Leise, 1979). Strathmann and Leise (1979) proposed that preoral cilia overtake particles in the latter part of the effective stroke and weakly adhere to them, pushing them faster than water. At the size and speed of ciliary movements involved in particle capture, viscous forces would dominate inertial forces (Vogel, 1981). Thus, at low Reynold's numbers the adhesion of small particles to rapidly moving cilia would probably not require an adhesive such as mucus. Rather than being involved in actual particle capture, materials released from the secretory cells instead may function in binding food particles, once already captured, into a string that travels along the adoral band, thus further ensuring the retention of food particles.

In *C. hastata,* the postoral band is comprised of simple cilia rather than compound cilia as observed in *O. edulis* (Erdmann, 1935; Waller, 1981). Since postoral cilia have been examined only in these two species it is difficult to generalize on the form of the postoral band among bivalve veligers. Whether the cilia are compound or simple may reflect on their role or efficiency in particle capture.

Compound cilia situated at the mouth region have not been previously described. Owing to their shape and location within the postoral band, we suggest their effective stroke is upward, toward the mouth, and therefore they are arranged in a single diaplectic row. Compound cilia arranged in row perpendicular to the plane of beat can flex more strongly than simple cilia and are capable of a greater tip velocity (Knight-Jones, 1954). These oral compound cilia probably help force food particles toward the mouth as they travel posteriorly along the food groove. Waller (1981) noted a postoral tuft in *O. edulis* which he described as rigid simple cilia, distinct from postoral cilia. He suggested the postoral tuft is sensory and may facilitate streaming of excess mucus and food posteriorly. Oral compound cilia described above are located between the postoral tuft and the mouth of *C. hastata.* Possibly these oral compound cilia serve a sensory function in selecting or rejecting food particles before they enter the mouth.

Luminal spaces within each primary gill filament also have been observed in *C. virginica* (Galtsoff, 1964; Elston, 1980) and *M. edulis* (Bayne, 1971). However, we were unable to observe any connection of these spaces to each other or with other vascular tissue. Elston (1980) noted that the spaces within the gill filaments—as well as other vascular tissues—may contain wandering amoeboid cells, suggesting a possible connection between luminal spaces. Prytherch (1934) observed blood cells circulating to the base of the gill filaments.

Structure of the larval eyespots in *C. hastata* is similar to that as described by Bayne (1971) for *M. edulis,* a cup-shaped group of small pigment granules deposited in a single cell. Galtsoff (1964) described the eyespots of *O. edulis* as consisting of several pigmented cells arranged in a circle around a transparent lens. Hickman and Gruffydd (1971) observed several pigment cells surrounding a lens, similar to Galtsoff, but noted that the granules were in the shape of a cup. There is little doubt that the eyespots are functional sensory organs in the larval stage; Galtsoff (1964) described nerve tracts leading to the eyespots of *O. edulis* and Bayne (1964) demonstrated that the appearance of the eyespots coincided with a change in phototactic behavior in *M. edulis* larvae.

Bivalve larval development generally is considered to be similar among species. Superficially, pectinids show little variation in development. With the exception of *Equichlamys bifrons* (Dix, 1976), all species investigated have a small egg (60–80 μm) and a planktotrophic larval stage. Duration of larval development varies with temperature and diet, but generally larvae reared at 15–18°C metamorphose 20–35 days after fertilization. Yet, as recent studies on larval hinge morphology (Lutz *et al.*, 1982) have demonstrated, there are subtle differences between species, enough to make them distinguishable from each other. In this study we have noted several differences in the morphology of *C. hastata* to that reported in the literature. Of particular interest are the unique compound cilia situated at the mouth region, the presence of a ligament pit in larval valves, and the observation that the postoral band is comprised of simple cilia. Examination of other bivalve larvae may reveal other structures previously unrecorded and we may begin to appreciate subtle morphological differences in larvae that are otherwise superficially similar.

Acknowledgments

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Development of Nerve Cells in Hydrozoan Planulae:
I. Differentiation of Ganglionic Cells

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Abstract. The cytomorphosis of ganglionic cells in hydrozoan planulae of *Halocordyle disticha* is described at the fine-structural level. Ganglionic cells arise from undifferentiated interstitial cells (I-cells). I-cells originate at 8 hours postfertilization as a central core of cells in the endoderm. Such I-cells possess a centrally located nucleus with one to several tiny nucleoli, a few segments of rough endoplasmic reticulum, a few mitochondria, electron-dense granules, and free ribosomes. Interstitial cells destined to form ganglionic cells migrate as single cells through the mesoglea to the base of the ectoderm where they can divide and subsequently differentiate. Early stages of neural differentiation are characterized by the enlargement of the I-cell nucleoli, the loss of electron-dense granules, and the appearance of a Golgi complex, numerous mitochondria, and microtubules. Next, neurites grow out from both sides of the ganglionic cell body and join neurites from adjacent ganglionic cells to form an extensive neural plexus just apical to the mesoglea in the planula. Neurites are rich in mitochondria and microtubules and extend in both a longitudinal and transverse direction with respect to the planular anterior-posterior axis. Some neurites extend down from the neural plexus and abut the mesoglea. The last phase of ganglionic cell development occurs when electron-dense droplets appear within the region of the Golgi and eventually in the neurites. Neurites contain both electron-dense droplets and dense cored vesicles located in clusters at specific intervals along their length. Such droplets and vesicles are often found in close association with the mesoglea in neurites which contact the mesoglea. Two morphological types of ganglionic cells are identifiable at the fine-structural level: a light ganglionic cell and a dark ganglionic cell. Light ganglionic cells possess an electron-lucent cytoplasm and fewer mitochondria than dark ganglionic cells. Dark ganglionic cells have an electron-dense cytoplasm and their cell bodies are more oblong than the rounder cell bodies of light ganglionic cells. Light ganglionic cells comprise the majority of the planular ganglionic population.

Neural differentiation begins in the planular ectoderm at 24 hours postfertilization and continues throughout larval development. The neural plexus is present at 24 hours and increases in size (*i.e.*, number of neurites) and complexity as animals mature. Ganglionic cells are distributed along the entire length of the planula and steadily increase in number as planulae age. All stages of ganglionic cell differentiation are found throughout planular development (24–96 hours postfertilization). Ganglionic cells of the planula show combined morphological features of interneurons and neurosecretory cells, suggesting they are multifunctional neurons.

Introduction

Cnidarians possess the most primitive nervous systems of all metazoans. Examination of the neurobiology of these animals is important because studying such lower phyla may help us understand: 1) the principles on which nervous systems work; 2) important neural similarities and differences among animal groups; and 3) the genesis and early evolution of the nervous system.

Hydrozoan planulae provide excellent developmental systems in which to examine patterns of neural differentiation (Martin and Archer, 1986). Ultrastructural examination of the nerve elements in mature planulae of four species of hydrozoans, *Halocordyle disticha* (*Pennaria tiarella*) (Martin and Thomas, 1980), *Mitrocomella polydiademata* (Martin *et al.*, 1983), *Hydractinia echinata* (Weis *et al.*, 1985), and *Phialidium gregarium* (Thomas *et al.*, 1987) indicates that the hydrozoan planular nervous system is composed of at least two cell types, sensory cells and ganglionic cells. Sensory cells extend from the free surface of the planula to the mesoglea and are

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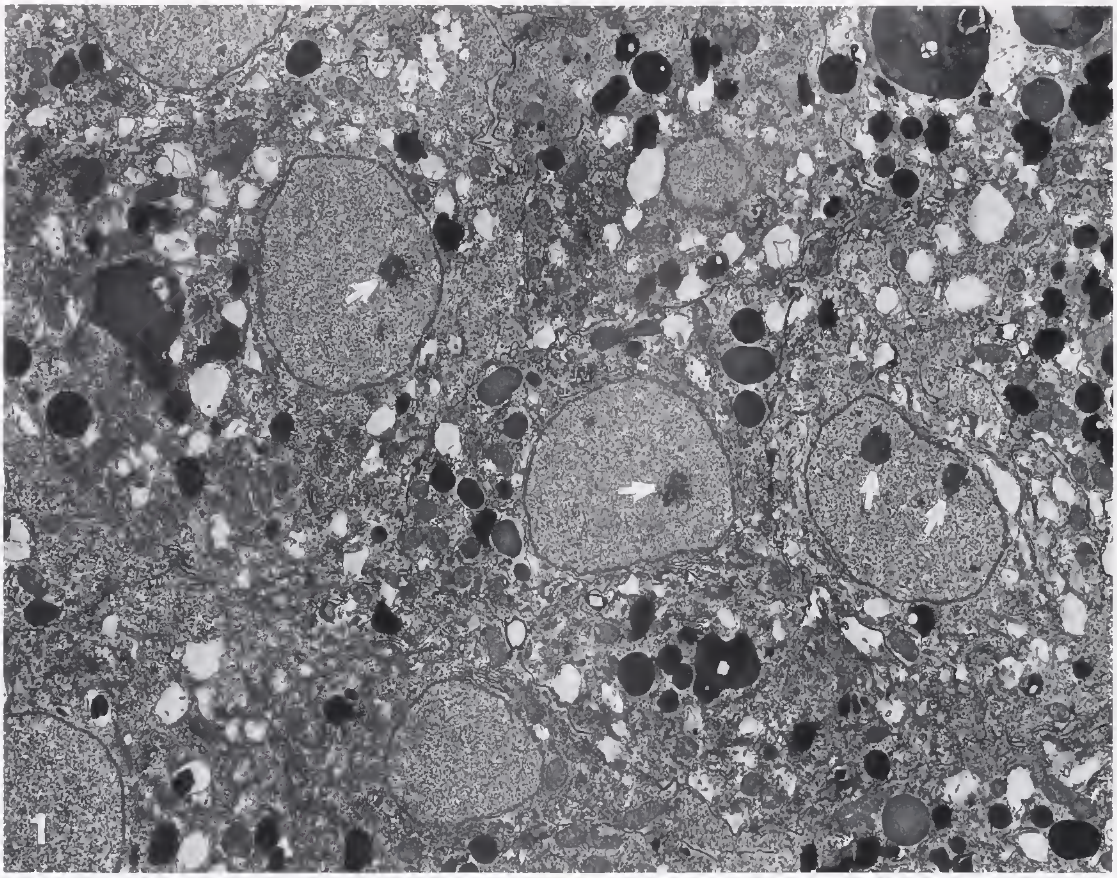
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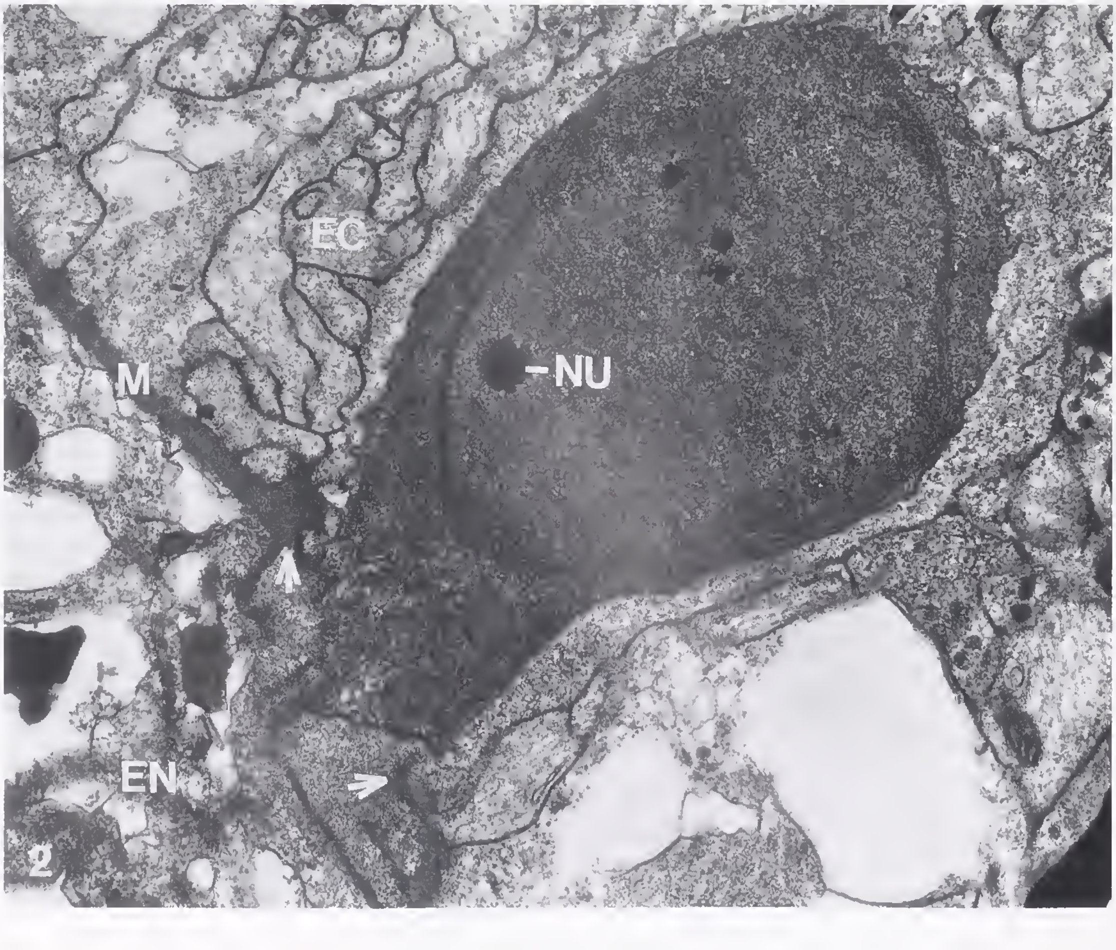
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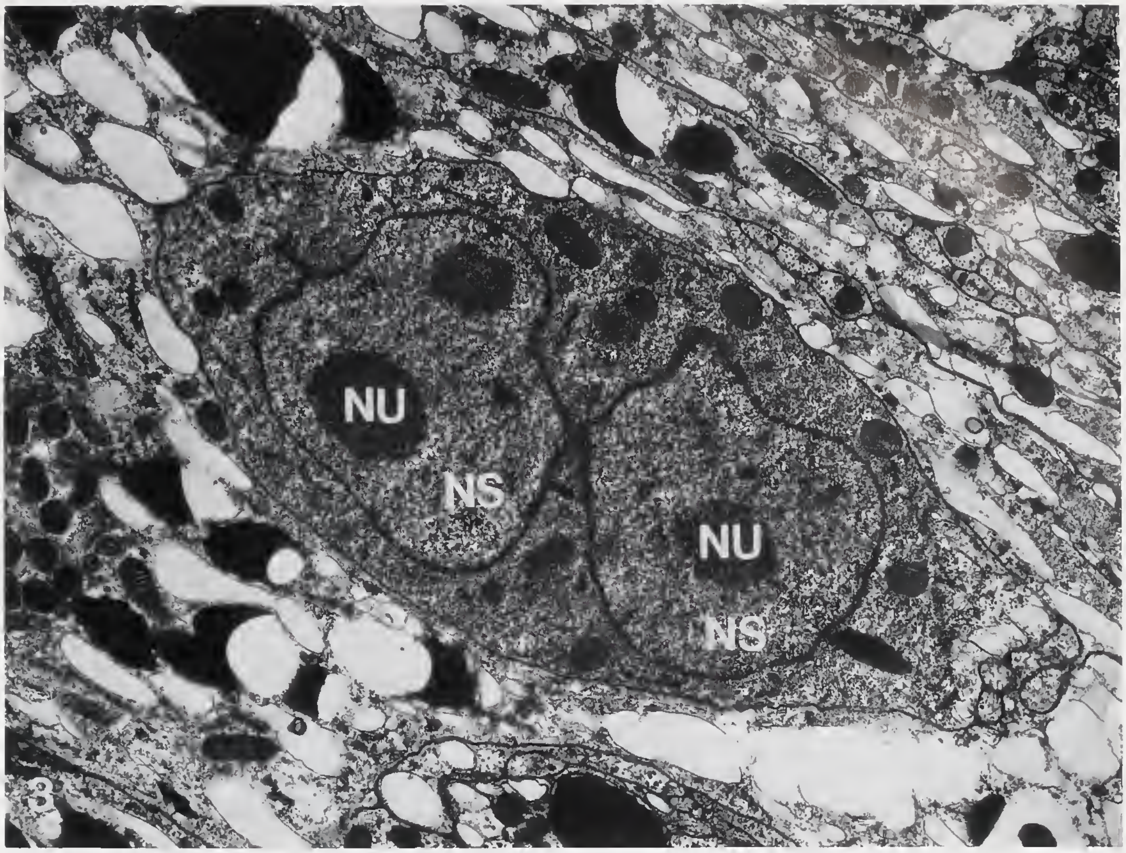

Figure 3. Dividing "neural" interstitial cell at the base of the ectodermal epithelium. Once such divisions are completed the progeny begin to differentiate into ganglionic cells. Such ganglionic cells grow neurites in between the cell bodies causing the cell bodies of the progeny to move apart. NS, nucleus; NU, nucleolus. ×11,670.

characterized by a single apical cilium and numerous cytoplasmic microtubules and neurosecretory droplets. Martin and Thomas (1980) and Thomas *et al.* (1987) demonstrated that planular sensory cells are derivatives of the ectodermal epithelium. Ganglionic cells are found at the base of the ectoderm and possess neurites rich in microtubules and mitochondria. Ganglionic cells arise from undifferentiated interstitial cells (Martin and Thomas, 1981a, b). Surprisingly, the cytomorphosis of sensory cells (Martin, submitted) and ganglionic cells in planulae has never been described.

This study employs transmission electron microscopy to examine the differentiation of ganglionic cells in hydrozoan planulae of *Halocordyle disticha.* Beginning with the young interstitial cell and ending with the fully formed ganglionic cell and its neurites, this work presents the first comprehensive morphological description of ganglionic cell differentiation and genesis of the neural plexus in the planula, identifies two morphological types of ganglionic cells in the planula, and provides morphological evidence suggesting that larval ganglionic cells are multifunctional neurons.

Materials and Methods

Colonies of the marine hydrozoan *Halocordyle disticha* were collected from pier pilings in Morehead City, North Carolina. Fronds from male and female colonies

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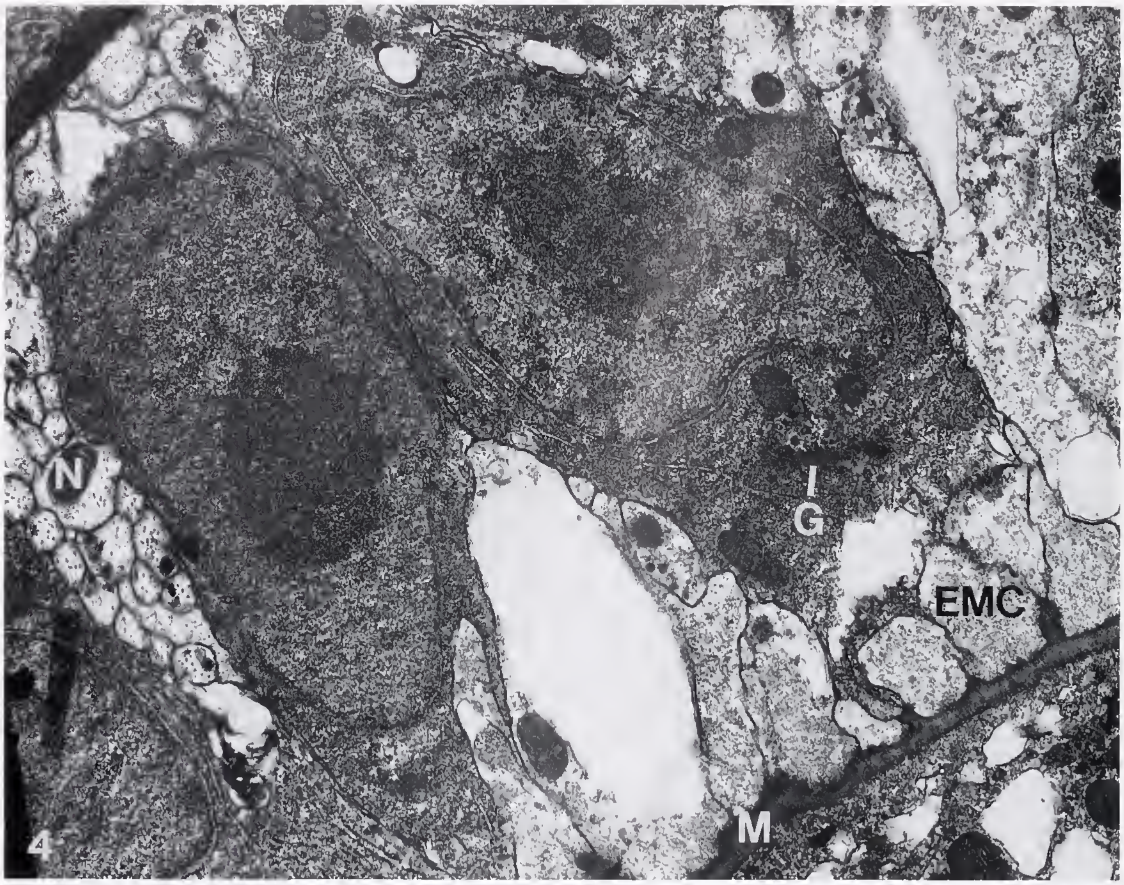
Figure 1. Interstitial cells in the endoderm of an 8 hour embryo. These cells are undifferentiated cells that contain a centrally located nucleus with one to several nucleoli (arrows). A few segments of rough endoplasmic reticulum, a few mitochondria and electron-dense granules, and ribosomes occupy the cytoplasm of the cell. ×5700.

Figure 2. Migrating interstitial cell in the mesoglea region. The cell has a trailing pseudopod and the mesoglea is disrupted (arrows) in the region of cell contact. Examination of serial sections confirms the broken nature of the mesoglea. EC, ectoderm; EN, endoderm; M, mesoglea; NU, nucleolus. ×21,000.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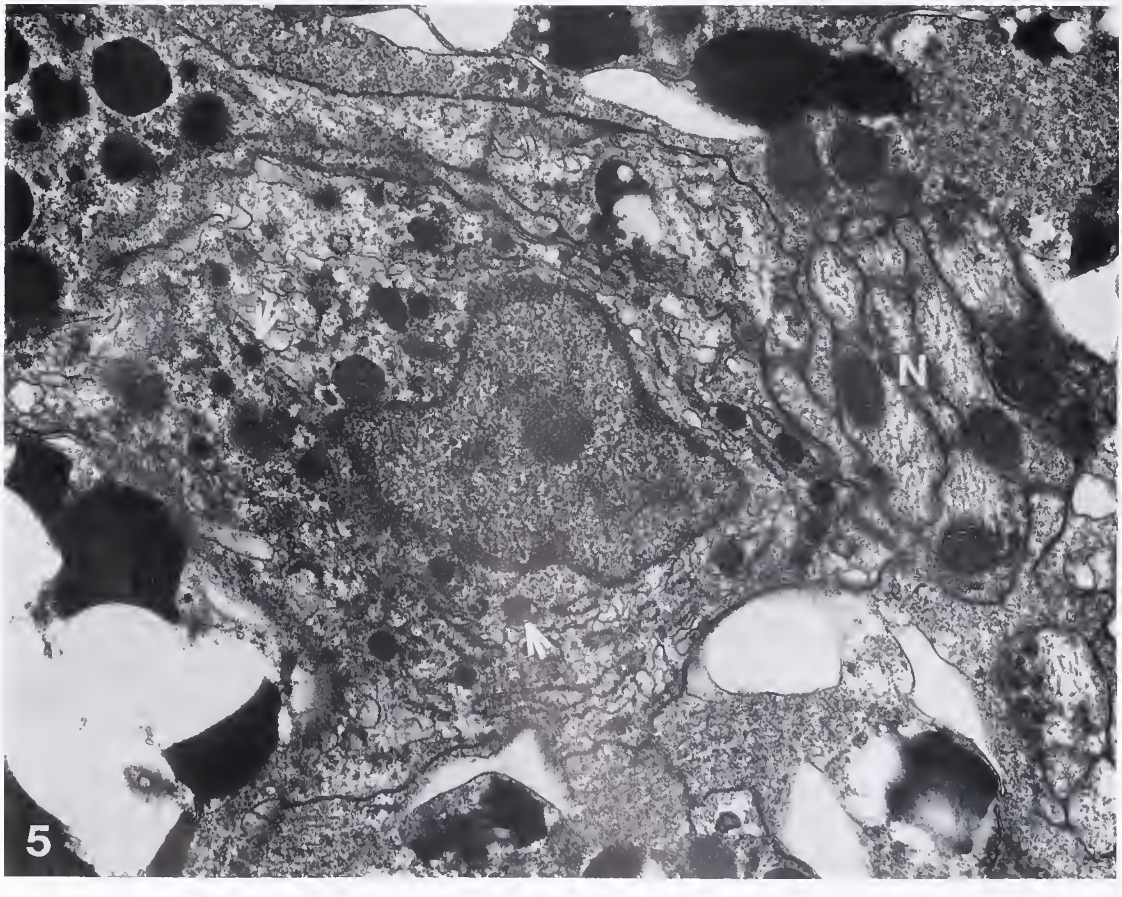
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were placed together in large finger bowls of filtered seawater. Bowls were placed in the dark at 6:00 pm and returned to the light at 9:00 pm. Shortly after exposure to light, early cleavage embryos appeared in the bottoms of the dishes. Embryos were collected, placed in small finger bowls of filtered seawater, and reared at 23°C.

Eight-hour embryos and 16-, 24-, 48-, 72-, and 96-hour planulae were processed for transmission electron microscopy. Samples were fixed for 1 hour in 2.5% glutaraldehyde, pH 7.4, in 0.2 *M* phosphate buffer. They were postfixed for 1 hour in 2% osmium tetroxide, pH 7.2, in 1.25% sodium bicarbonate. Specimens were dehydrated in an ethanol series, infiltrated, and embedded in Spurr's embedding media. Blocks were serially thin-sectioned on a Porter-Blum MT-2B ultramicrotome, placed on 150-mesh copper grids, and stained with 3.5% uranyl acetate in ethanol followed by lead hydroxide. Grids were examined and photographed with a Hitachi H-600 transmission electron microscope.

Results

In *Halocordyle,* ganglionic cells form from undifferentiated interstitial cells (Martin and Thomas, 1981a, b). The interstitial cells arise during gastrulation (8–10 hours postfertilization) as a central core of cells located in the endoderm (Fig. 1). These young interstitial cells are round, measuring *ca.* 7 μm in diameter. These early I-cells contain a centrally located nucleus with one to several tiny nucleoli. Free ribosomes, a few mitochondria, and a few segments of rough endoplasmic reticulum may fill the cytoplasm of the I-cell. Small electron-dense granules may also be present in the cytoplasm. As interstitial cells mature, these electron-dense granules are lost and the nucleoli greatly enlarge.

Interstitial cells destined to form ganglionic cells emigrate as single cells from the endoderm to the ectoderm from all positions along the anterior-posterior axis of the planula. (Fig. 2). Such migrating I-cells possess a trailing pseudopod. As I-cells cross the mesoglea, the mesoglea appears to break in the region of transection (Fig. 2). Once I-cells have moved into the ectoderm the broken ends of mesoglea appear to reconnect. I-cells migrate to the base of the ectoderm, stop, sometimes divide, and subsequently develop morphological characteristics of ganglionic cells (Figs. 3–9). I-cells continually migrate to the base of the ectoderm throughout larval development. Cell bodies of fully differentiated ganglionic cells measure *ca.* 5 μm in diameter and are first detected in the ectoderm at 24 hours postfertilization. Ganglionic cells are distributed along the entire anterior-posterior axis of the planula. They increase in number as development proceeds and are found in the planula throughout its remaining larval life.

Once "neural" interstitial cells have migrated to the ectoderm they locate in the interstitial spaces just apical to the foot processes of the epitheliomuscle cells. These "neural" I-cells may undergo division in the ectoderm (Fig. 3) and then differentiate into ganglionic cells (Figs. 4–9). The nucleoli of the forming ganglionic cells enlarge and the nuclei exhibit condensed chromatin (Figs. 4–7). If multiple nucleoli are present in the nuclei of these forming ganglionic cells, they appear to eventually coalesce to form a single large nucleolus. A Golgi complex, segments of rough endoplasmic reticulum, mitochondria, and microtubules appear in the cytoplasm of the differentiating cell (Figs. 4–7). Shortly after these organelles appear, neurites begin to form (Figs. 4, 5). Such neurites grow out from both sides of the cell body and are rich in microtubules and mitochondria (Fig. 5). These growing neurites make contact with neurites from adjacent ganglionic cells thus forming a neural plexus at the base of the ectoderm. As the neurites are forming, electron-dense droplets appear within the rough endoplasmic reticulum and the Golgi region of the perikaryon (Fig. 5). Shortly after these droplets appear in the cell body region they also are seen in the neurites (Figs. 5, 6). These droplets appear not to be packaged in membranes. Membrane-bound dense cored vesicles are visible in the

Figure 4. Developing ganglionic cells at the base of the ectodermal epithelium of a 24 hour planula. Interstitial cells destined to form ganglionic cells migrate to the ectoderm and locate in the interstitial spaces just apical to the foot processes of the epitheliomuscle cells (EMC). Once in position, these I-cells insert themselves into a forming neurite plexus and develop morphological characteristics of ganglionic cells. Presumably the profiles of neurites (N) that are visible in this micrograph have arisen from interstitial cells which previously differentiated. Early stages of ganglionic cell differentiation include the appearance of a Golgi complex (G), a few segments of rough endoplasmic reticulum, and a few mitochondria in the cell cytoplasm. M, mesoglea. ×14,700.

Figure 5. Differentiating ganglionic cell in a 24 hour planula. The cytoplasm becomes filled with mitochondria and microtubules. Neurites (N) begin to grow out from the cell body in both a transverse and longitudinal direction. The last phase of neural differentiation is characterized by the appearance of electron-dense droplets (arrows) in the cytoplasm of the cell body and in the neurites (N). These droplets first appear in the region of the rough endoplasmic reticulum and then in the region of the Golgi. Droplets in the Golgi are smaller in diameter than in the rough endoplasmic reticulum suggesting that the material is compacted as it moves through the cytoplasm of the perikaryon. ×18,700.

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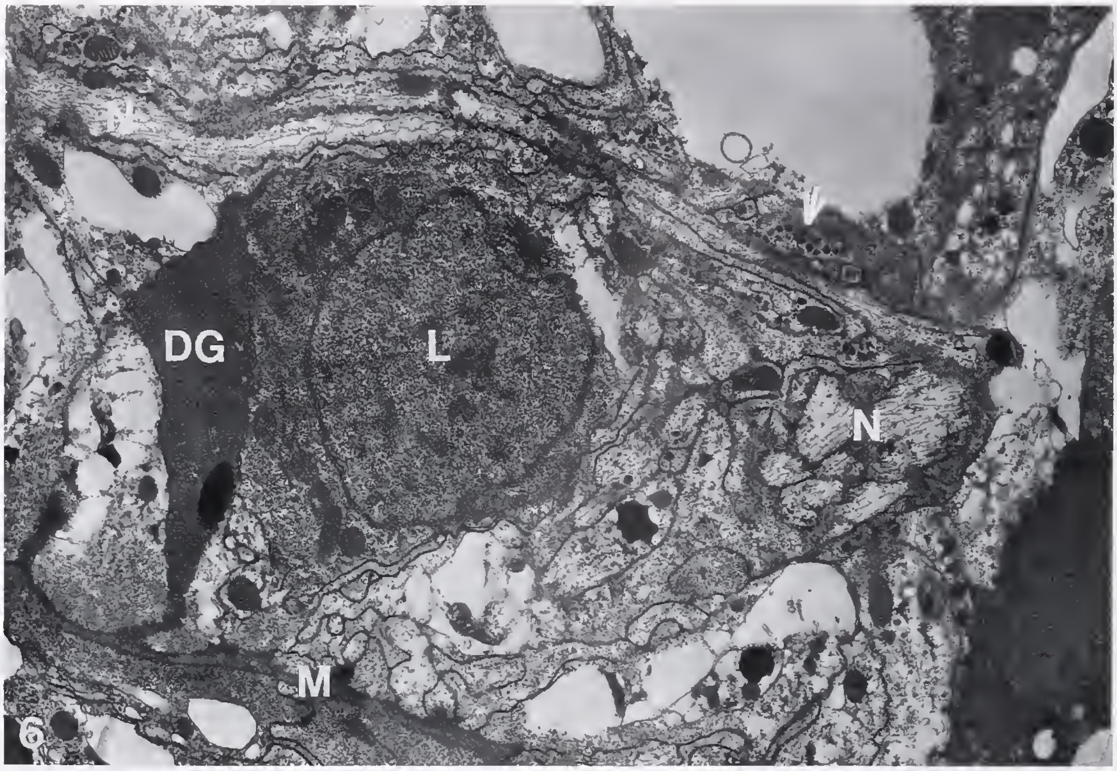

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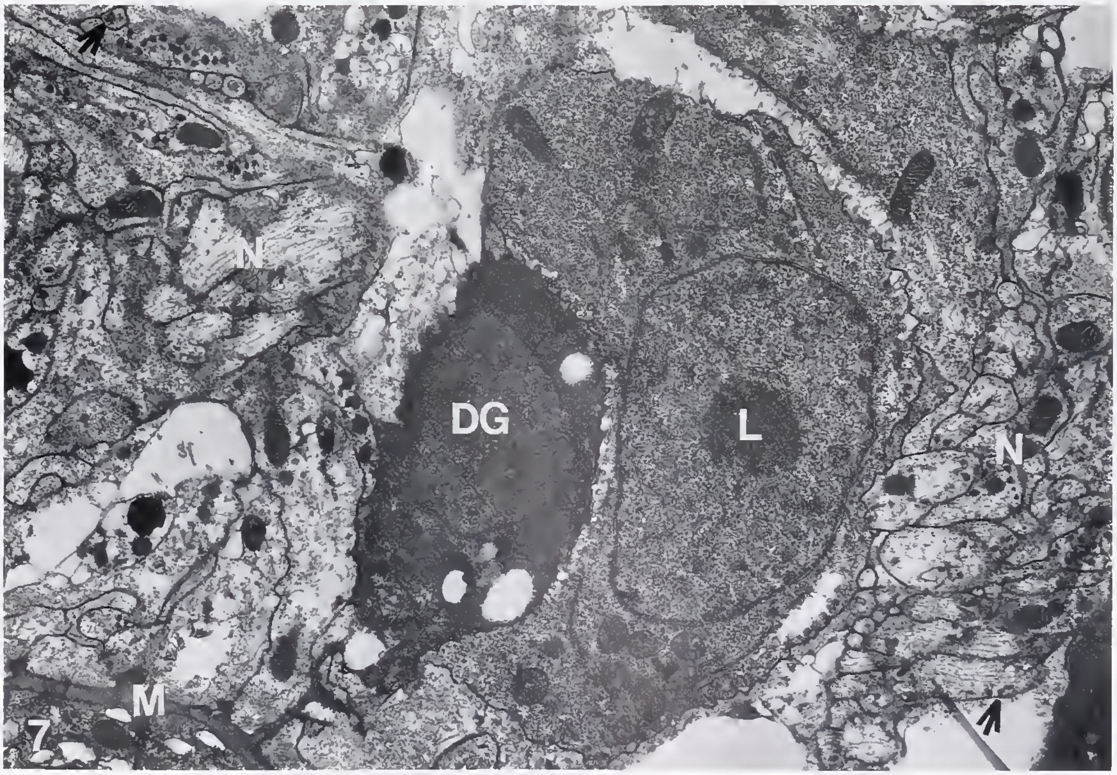
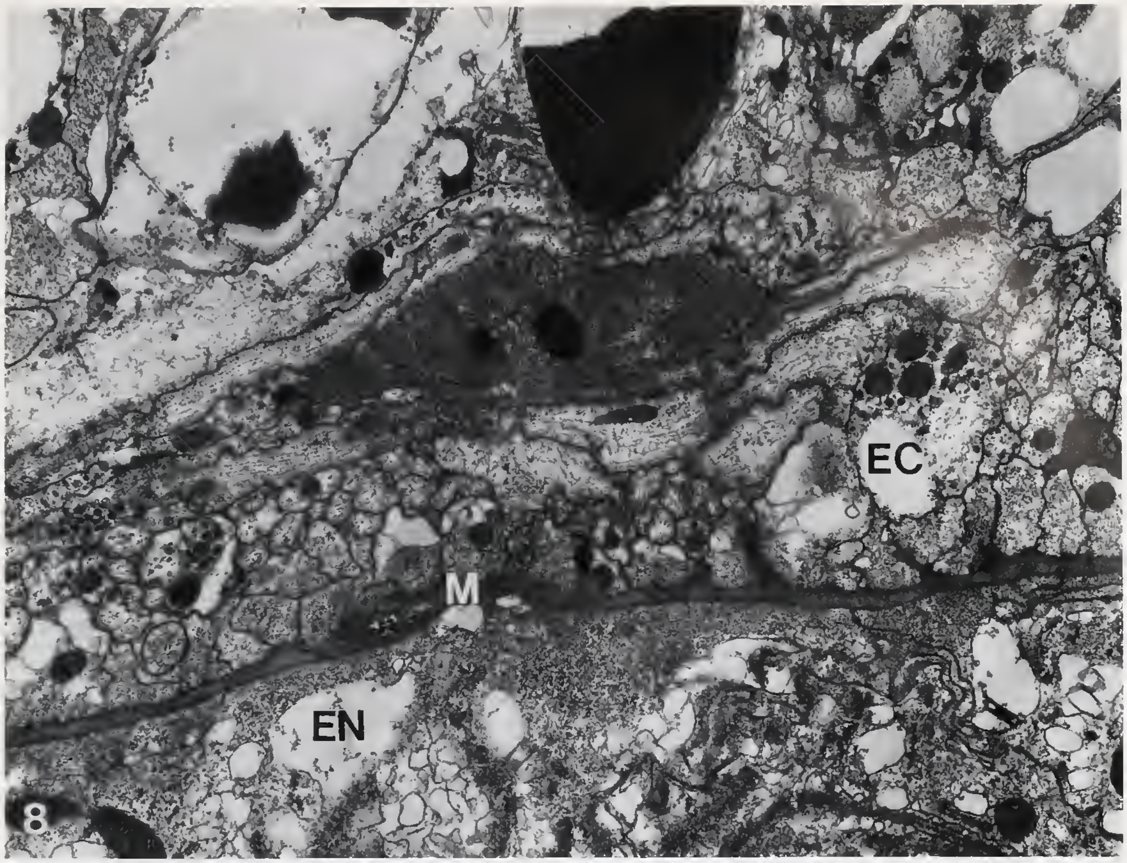


Figure 6. Dark and light ganglionic cells in a 24 hour planula. Light ganglionic cells (L) comprise the majority of the ganglionic cell population. They have an electron-lucent cytoplasm rich in microtubules and mitochondria. A portion of a dark ganglionic cell (DG) is visible in close association with the light ganglionic cell. Both cells contribute neurites (N) rich in microtubules, mitochondria, and electron-dense droplets (arrow) to the plexus. M, mesoglea. ×10,000.

Figure 7. Dark and light ganglionic cells at the base of the ectoderm of a 24 hour planula. Dark ganglionic cells (DG) are smaller than light ganglionic cells (L) and possess an abundance of mitochondria. Neurites (N) extend from both cells. Arrows, dense cored vesicles; M, mesoglea. ×10,000.



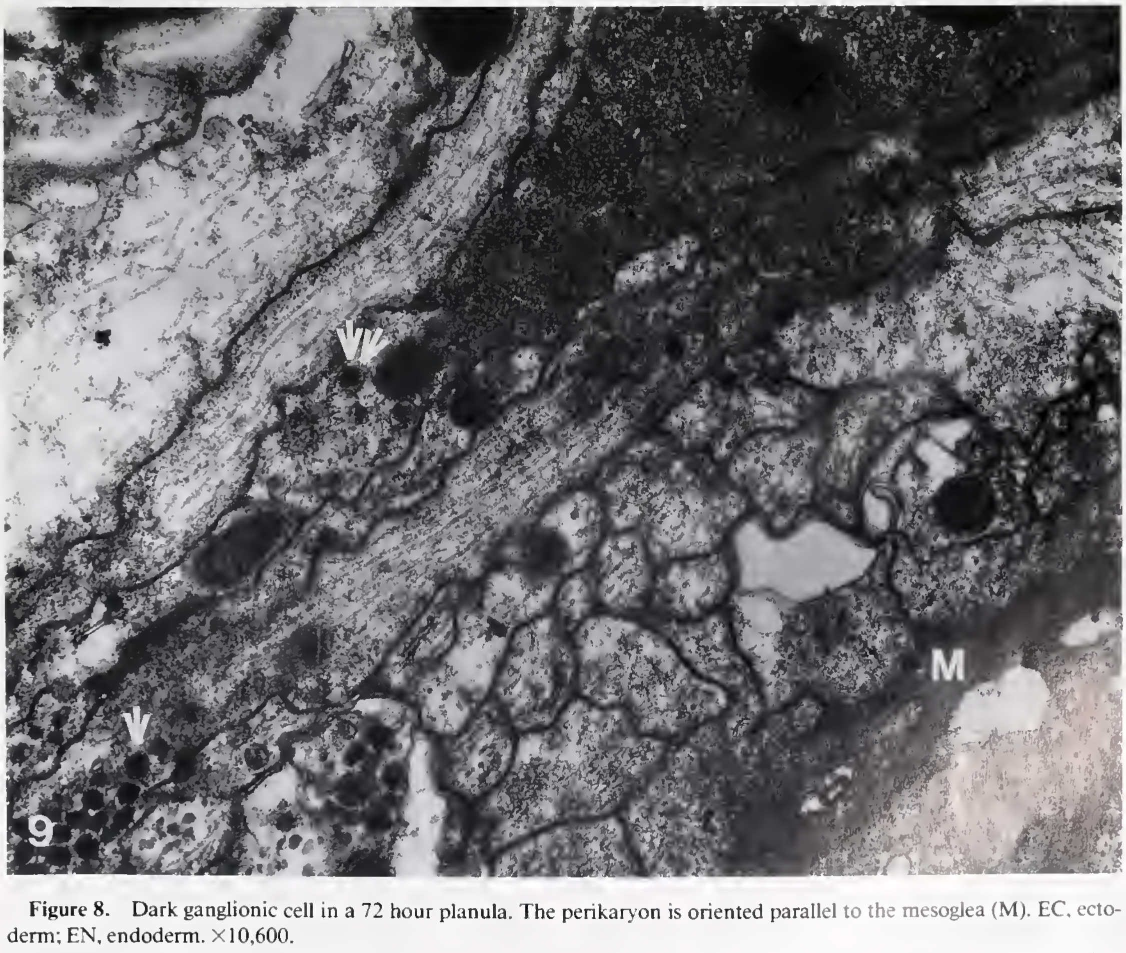


Figure 8. Dark ganglionic cell in a 72 hour planula. The perikaryon is oriented parallel to the mesoglea (M). EC, ectoderm; EN, endoderm. ×10,600.

Figure 9. Enlargement of the neurite region of the dark ganglionic cell seen in Figure 8. Both electron-dense droplets (single arrow) and dense cored vesicles (double arrows) fill the neurites of these dark cells. M, mesoglea. ×27,500.

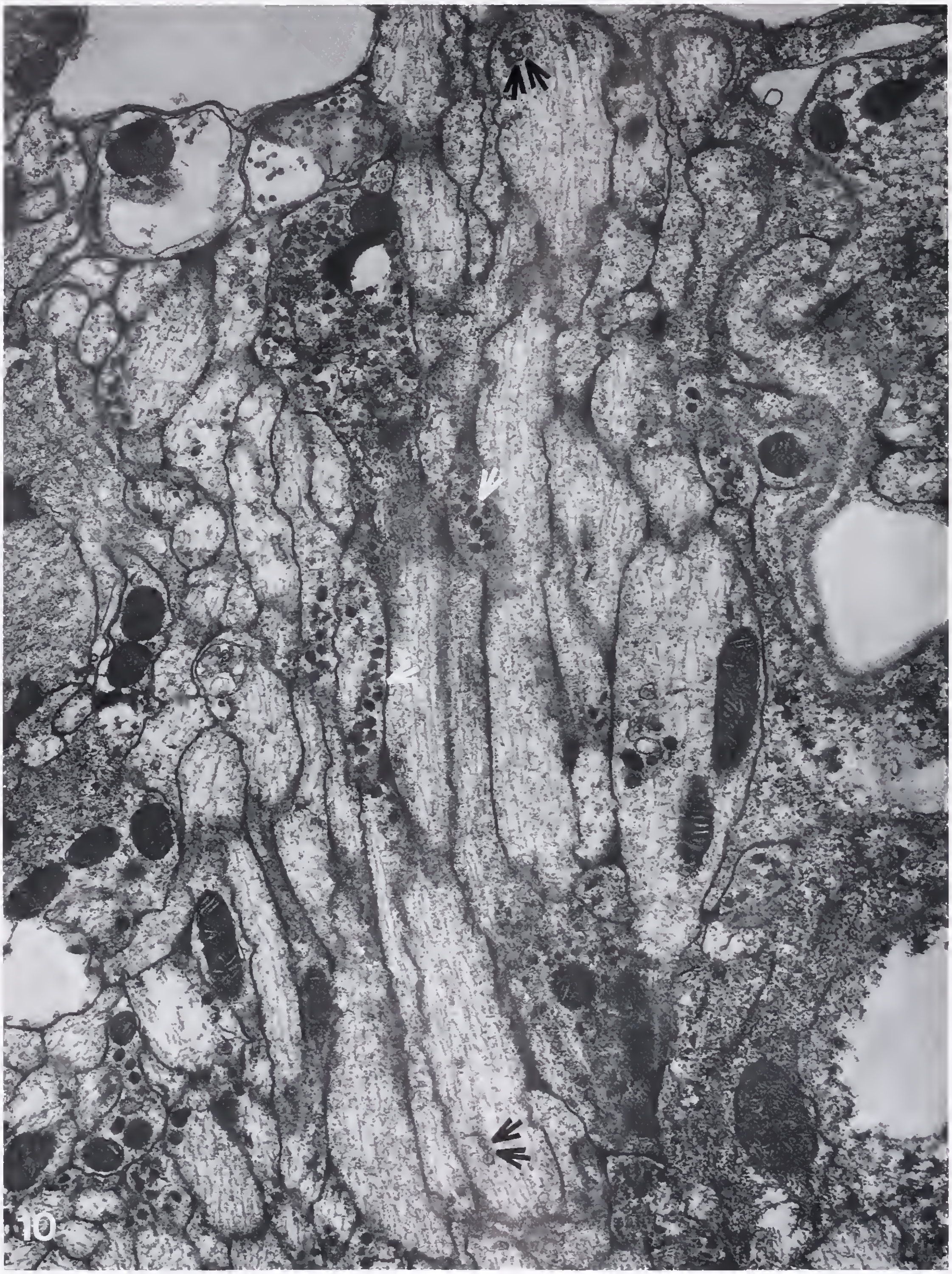
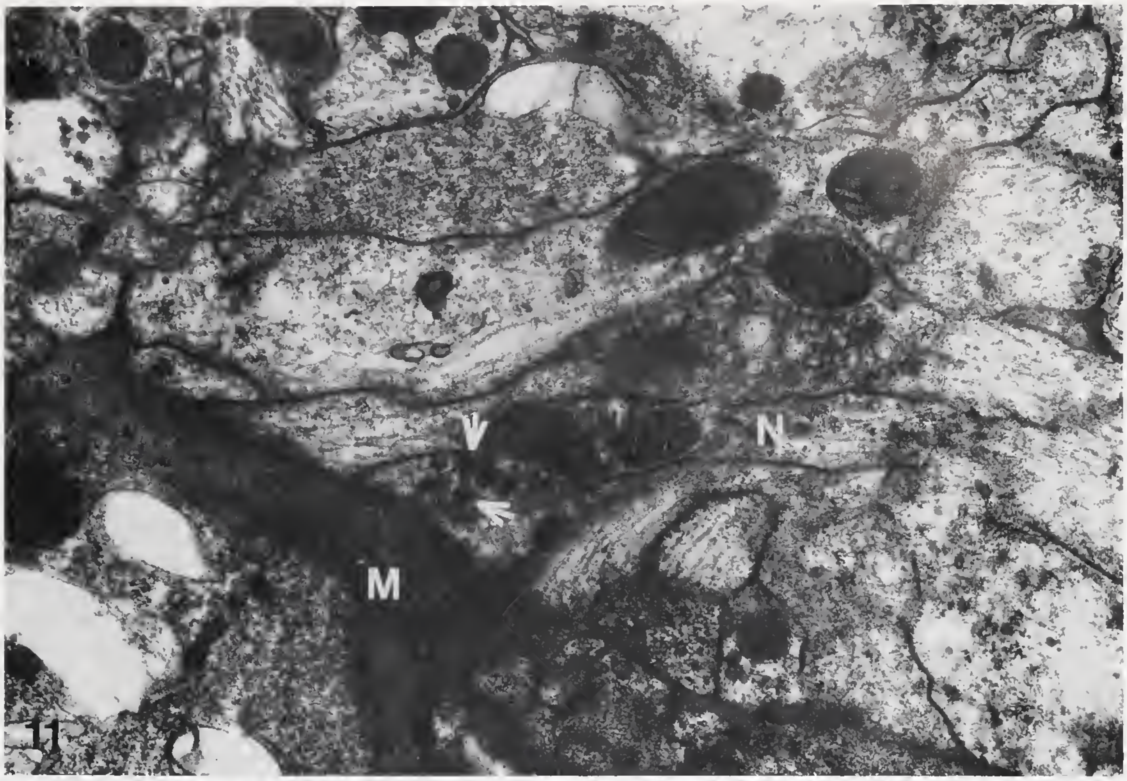


Figure 10. Well-developed nerve plexus at the base of the ectoderm of a 72 hour planula. Tracks of neurites rich in microtubules, mitochondria, electron-dense droplets (single arrow), and dense cored vesicles (double arrows) are conspicuous. These droplets and vesicles are located in clusters at certain positions along the neurites. ×18,000.



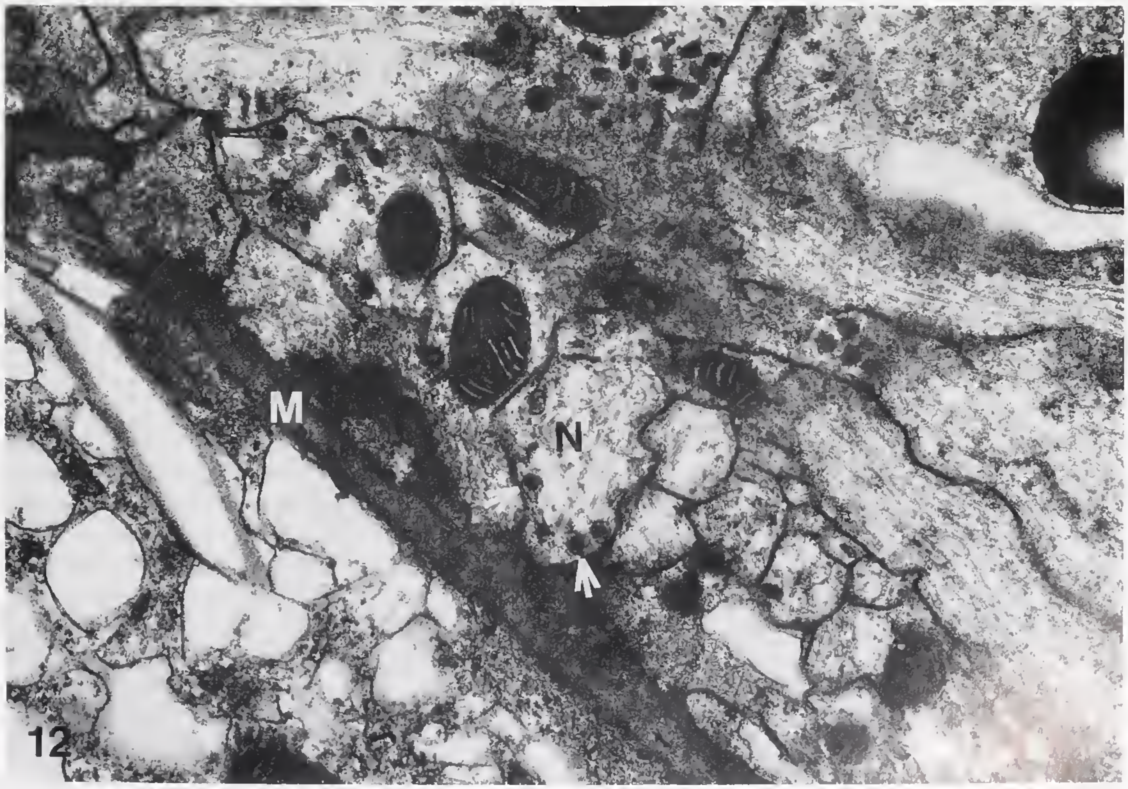


Figure 11. Neurites abutting the mesoglea (M) in a mature planula. Electron-dense droplets (arrows) of the neurites (N) are seen in close proximity to the mesoglea. ×27,500.

Figure 12. Dense cored vesicles (arrows) of neurites (N) in intimate association with the mesoglea (M). ×27,500.

neurites shortly after the appearance of the electron-dense droplets in the neurites. The fully differentiated ganglionic cell is characterized by a centrally located nucleus, a cytoplasm rich in microtubules and mitochondria, a cytoplasm containing a Golgi complex and electron-dense droplets and dense cored vesicles, and neurites extending from both sides of the cell body (Figs. 6, 7).

Two morphological types of ganglionic cells are found in the planula: a light ganglionic cell and a dark ganglionic cell (Figs. 6–9). Both types of ganglionic cells differentiate in the same manner (*i.e.*, pass through the same morphological stages of differentiation) as described above. Light ganglionic cells have an electron-lucent cytoplasm, possess fewer mitochondria than dark ganglionic cells, and their cell bodies are rounder than those of dark ganglionic cells (Fig. 7). Dark ganglionic cells possess an electron-dense cytoplasm, a small nucleus, and numerous mitochondria (Figs. 7, 8). The cell bodies of dark ganglionic cells are more oblong than those of light ganglionic cells, with their oblong axes oriented parallel to the mesoglea (Fig. 8). Light ganglionic cells comprise the majority of the ganglionic cell population and first appear at 24 hours postfertilization. A few dark ganglionic cells are present in 24 hour planulae. However, the majority of dark ganglionic cells arise later in planular development (48–96 hours postfertilization) just prior to metamorphosis. Light ganglionic cells are distributed along the whole anterior-posterior axis of the planula, whereas dark ganglionic cells are found in the head and anterior mid-sides of the planula. Both types of ganglionic cells contribute neurites to the neural plexus (Figs. 9–12).

The ectodermal neural plexus, located apical to the mesoglea, consists of transversely and longitudinally oriented processes which are present along the entire length of the planula (Figs. 10–12). The plexus of neurites is detected as early as 24 hours postfertilization and increases in size (*i.e.*, the number of neurites increase) and complexity as the planula matures. Electron-dense droplets and dense cored vesicles are abundant in the plexus and are found in clusters in specific regions of the neurites and are not evenly distributed along the entire length of the neurites (Fig. 10). Neurites extend down from the plexus and abut the mesoglea (Figs. 11, 12). In the region of neurite contact with the mesoglea electron-dense droplets and dense cored vesicles are found. The neural plexus in the head region of the planula is more extensive than the more posterior neural plexus, however there does not appear to be an accumulation of ganglionic cells in the head region.

Discussion

Hydrozoan planulae provide good embryonic systems in which to examine neural differentiation. In many planulae the entire nervous system forms within a period of a few days (Martin and Thomas, 1980; Martin *et al.* 1983). In *Halocordyle disticha* planulae, fully differentiated ganglionic cells first appear in the ectoderm as early as 24 hours postfertilization. These ganglionic cells are derivatives of endodermal interstitial cells which arise between 8–10 hours postfertilization. Ganglionic cell differentiation occurs rather quickly (at most 14–16 hours are needed for the first ganglionic cells to appear in the ectoderm once interstitial cells form); it is also easily characterized using transmission electron microscopy: appearance of a Golgi complex, mitochondria, microtubules, droplets, vesicles, and neurites. By 24 hours a neural plexus composed of neurites from both ganglionic cells and from sensory cells is prominent just above the mesoglea (Martin, unpub. obs.). This plexus increases in diameter and acquires more droplets and vesicles, and more ganglionic cells differentiate and insert their neurites into the plexus as the planula matures. Thus by 48–96 hours the nervous system of the planula (sensory cells and ganglionic cells) is quite extensive and well-organized.

Two types of ganglionic cells are morphologically identifiable at the fine-structural level in *Halocordyle disticha* planulae: a light ganglionic cell and a dark ganglionic cell. Light ganglionic cells have been observed in other hydrozoan planulae; the dark ganglionic cell has not been previously identified (Martin *et al.*, 1983). A few dark ganglionic cells are found in 24 hour planulae, however the majority of dark ganglionic cell differentiation occurs later in planular life (just prior to metamorphosis). The fact that dark ganglionic cells increase in number just prior to metamorphosis may indicate their involvement in the metamorphic process.

Martin and Thomas (1981b) proposed a neural mechanism of control of metamorphosis in hydrozoan planulae. They suggested that sensory cells of the planula perceive the necessary stimulus for attachment and metamorphosis and convey this information to ganglionic cells. In turn, ganglionic cells via either neurosecretion (chemical) and/or neurotransmission (electrical) transmit the metamorphic stimulus to the remaining larval cell types. The morphology and architecture of the planular nervous system of *Halocordyle disticha* support Martin and Thomas's (1981b) theory. Sensory cells of the planula make contact with the external environment and also feed neurites into the ganglionic plexus (Martin, unpub. obs.). Hence it is easy to visualize an integrative neural system in the ectoderm. The endoderm, however, lacks neural elements. This study demonstrates that ectodermal neurites make contact with the mesoglea. Furthermore, in the region of such contact electron-dense droplets and dense cored vesicles are found. If ganglionic cells function in neurotransmission and/or neurosecretion, then one can now visualize how a metamorphic stimulus could also be propagated rather quickly to the endoderm. The vesicles in contact with the mesoglea could be discharged, releasing their products into the mesoglea and ultimately to the endodermal cells. Thus both ectodermal and endodermal larval cells would be able to respond rapidly to an appropriate metamorphic stimulus, even though there are no nerves in the planular endoderm.

Ganglionic cells (both dark and light) of the planula exhibit combined morphological features of interneurons and neurosecretory cells and bear some resemblance to the sensory-motor-interneurons (ganglion cells) described for adult *Hydra* (Westfall, 1973). Neurites of planular ganglionic cells make contact with neurites of adjacent ganglionic cells and with neurites of sensory cells (Martin, unpub. obs.) and hence resemble interneurons. Planular neurites also abut the mesoglea. Furthermore, planular ganglionic cells and their neurites contain numerous droplets and vesicles. Recently, Martin (1987) and Kolberg and Martin (1987) demonstrated neuropeptide-like substances and catecholamines in association with some of the ganglionic cells and their neurites. Hence, the planular ganglionic cell has combined features of an interneuron and a neurosecretory cell. Westfall and Kinnamon (1978) proposed that such multifunctional features combined into a single neuron might indicate that the neuron is a primitive stem cell from which evolved more specialized nerve cells found in higher animals. The present study suggests that the planula, the larval form of cnidarians, contains multifunctional neurons.

Acknowledgments

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Spermatogenesis and Sperm Storage in the Testes of the Behaviorally Distinctive Male Morphotypes of *Macrobrachium rosenbergii* (Decapoda, Palaemonidae)

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Abstract. Adult males of the freshwater prawn *Macrobrachium rosenbergii* differentiate into three morphotypes. Each morphotype develops in sequence in the adult male population from small males through orange claw males to dominant blue claw males. Small males and blue claw males are sexually active, while orange claw males represent a sexually inactive intermediate stage engaged in somatic growth.

To further examine these behavioral characteristics on a physiological level, testes from each of the morphotypes were cultured *in vitro*. The rate of [^{3}H]-thymidine incorporation, representing DNA synthesis, and the amount of sperm released into the culture media were recorded. In parallel, the relative wet weight of testes from the different morphotypes were recorded and histological preparations were examined.

The relative weight of the testes from small males was significantly greater than in the other morphotypes. The testes of small males were both active in spermatogenesis and contained a large amount of mature sperm. Transition to the orange claw morphotype is marked by spermatogenically active testes, characterized by a multilayered spermatogenic zone and a high rate of thymidine incorporation. The testes of the orange claw morphotype develop into organs containing mainly spermatocytes. The fully differentiated orange claw male will transform by a metamorphic molt into the sexually active blue claw male. Almost no spermatogenic activity was recorded in the testes of the blue claw males. The testes of the blue claw males contained, almost exclusively, mature sperm.

The anatomical, physiological, and histological status of the male morphotypes are closely associated with the growth pattern and behavioral attributes of the morphotypes as they differentiate through the developmental pathway from the small male through the orange claw to the blue claw male morphotype.

Introduction

Among mature males of the freshwater prawn, *Macrobrachium rosenbergii*, three distinctive morphological types appear: small males, orange claw males, and blue claw males (Ra'anan, 1982). The male morphotypes differ from each other in their claw color, relative claw length, and spination (Kuris *et al.*, 1987) as well as in their reproductive behavior (Ra'anan and Sagi, 1985) and growth rates (Ra'anan and Cohen, 1985). Two of the morphotypes—the blue claw and small males—represent two alternative mating strategies. Large blue claw males are able to court actively and protect females prior to and during mating. Small males engage in a form of 'sneak copulation' consistent with their small size and high mobility. Orange claw males are a rapidly growing intermediate phase from the small male to the blue claw morphotypes. Orange claw males infrequently engage in reproductive activity (Ra'anan and Sagi, 1985).

The male reproductive system in *Macrobrachium rosenbergii* consists of a pair of testes, a pair of vasa deferentia, and a pair of genital pores. The testes are elongated structures, united at their anterior end, which lie between the dorsal surface of the hepatopancreas and the heart. Each testis consists of a large number of lobules or cylinders compactly held together by connective tissue (Pat-

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wardham, 1937; Sreekumar *et al.*, 1982). Spermatogenic cells and sustentacular cells constitute the two major types of cells within the testicular cylinder. Mature spermatoza are confined to a lumen within the testicular cylinder, described as a blind-ending cyst by Dougherty and Sandifer (1984).

Previous work showed that the relative weight and anatomical development of the reproductive system correlate with the morphological and behavioral changes in males occurring during the morphotypic differentiation process (Sagi and Ra'anan, 1988). Since the three male morphotypes are reproductively distinct, it was of interest to examine the difference in their gonadal structure and activity. The present study focuses on differences among male morphotypes in the testes *i.e.*, gonado-somatic index and gonad activity, as observed *in vitro* by sperm release and [^{3}H]-thymidine incorporation into DNA in the testes. In addition, the histological structure of the testes from different male morphotypes was studied. We found that the structural, physiological, and biochemical properties of the testes are closely associated with the morphological and reproductive states of these morphotypes.

Materials and Methods

Selection of prawns

Male prawns were selected from a single aged population after 150 days of growth in earthen ponds at the Dor Aquaculture Research Station, Israel. Prawns were divided into five categories according to coloration, second pereiopod (claws) shape and spination, and propodus and carapace length (Ra'anan, 1982; Ra'anan and Cohen, 1985; Kuris *et al.*, 1987). Carapace and propodus length were measured with a caliper to 0.1 mm. Carapace length was defined as the distance from the posterior margin of the right orbit to the midline of the posterior margin of the carapace. The propodus, with the joint flexed, was measured along the lateral face from the proximal lateral condyle to the distal tip.

Fifteen prawns of each category were selected for study. Small males, weak orange claw, strong orange claw, and blue claw forms were selected according to the methods described by Kuris *et al.* (1987). Pretransforming strong orange claw males were selected according to the characteristics described by Sagi and Ra'anan (1987).

Gonado-somatic Index (GSI)

The weight of the prawns and the wet weight of the gonads were measured, after drying them thoroughly on filter paper, using a precision balance (±1 mg). The GSI was calculated as follows:

$$\mathrm{GSI} = 100 \times \frac{\text{gonad wt.}}{\text{body wt.}}.$$

Organ culture ([^{3}H]-thymidine incorporation and sperm release)

The animals were dissected under sterile conditions upon arrival in the laboratory. The testes were clipped off the sperm duct and were incubated intact in 2 ml culture medium (12 well, cell culture plates, Nunc). The medium consisted of Dulbeco's Modified Eagles Medium (DMEM) containing 1 m*M* glutamine and 3.2 g $NaHCO_3$ which was adjusted to *M. rosenbergii* osmolarity by supplementing the following salts: KCl (160 mg/l), $MgCl_2 \cdot 6H_2O$ (240 mg/l) and NaCl (3.16 g/l). The salts were added according to the concentrations found in hemolymph of *Macrobrachium* (Stern, 1985) and pH was adjusted to 7.6 with 1 *N* NaOH (Nagamine *et al.*, 1980). Radiolabelled [^{3}H]-thymidine was added as a tracer at zero time (amount: 2 μCi/ml, specific activity 52 Ci/mmol, New England Nuclear). After 24 hours of incubation at 25°C, the organs were removed from the culture dishes. Morphological and histological examinations, performed on some of the cultured organs at this stage, revealed no apparent signs of deterioration. Sperm remaining in the medium (probably released via the excised sperm duct opening) were counted using a hemocytometer. The tissue was homogenized at 1–2°C in 2 ml Tris HCl buffer 10 m*M*, pH = 7.4. The homogenate of the testes was incubated for an additional 5 min on ice after the addition of 2 ml 10% TCA solution and then filtered through fiberglass filters (GFC, Whatman). The papers were then rinsed once with cold 10% TCA solution and twice with 98% ethyl alcohol. The paper was dried then incubated in scintillation fluid (toluene:triton, 1:3, overnight at 25°C). Radioactivity was measured in a liquid scintillation spectrophotometer. Results are expressed in counts per minute per 10 milligram wet weight of testes.

Histological observations

From each morphotype, testicular samples from five individual prawns displaying unequivocal morphotypic characteristics were selected for histological study. The prawns were dissected and the testes were removed and fixed in Brodski fluid (Brodski, 1960). The samples were then embedded in paraffin, serially sectioned at 7 μm, and stained with Ehrlich's hematoxylin-eosin (Humason, 1967).

Statistical analysis

Analysis of variance (Sokal and Rohlf, 1981) was performed to analyze variation in: (a) the Gonado-somatic

Table I

The Gonado-somatic Index of different Macrobrachium rosenbergii *male morphotypes*

Morphotype	GSI ± SE
Small male	0.24 ± 0.04
Weak orange claw	0.10 ± 0.03
Strong orange claw	0.09 ± 0.01
Pretransforming orange claw	0.16 ± 0.03
Blue claw	0.14 ± 0.01

Index, (b) [^{3}H]-thymidine incorporated in counts per minute per 10 mg gonads, and (c) the amount of sperm released into the culture media per 10 mg gonads among the various male morphotypes. The SPSS (Statistical Package for the Social Sciences) was used for this analysis.

Results

Gonado-somatic Index

Table I shows the GSI, calculated as a percentage of total body weight. It is apparent that strong orange claw males are characterized by a significantly lower Gonado-somatic Index when compared to small males and blue claw males, 0.09 ± 0.01 *versus* 0.24 ± 0.04 ($P < 0.005$) and 0.14 ± 0.01 ($P < 0.05$), respectively. Among the orange claw males, the Gonado-somatic Index of the strong orange claw males does not differ significantly from the weak orange claw males, while it is significantly lower than that of the pretransforming strong orange claw males ($P < 0.05$).

[^{3}H]-Thymidine incorporation and sperm release by the gonad in vitro

The incorporation of [^{3}H]-thymidine into cultured cells measures the rate of DNA synthesis (Collins, 1977). Our preliminary studies showed that 70%–85% of [^{3}H]-thymidine incorporated into *M. rosenbergii* testes in organ culture was indeed found in the cellular DNA fraction. This was shown by inhibition of thymidine incorporation by hydroxyurea and by the lability of the [^{3}H]-thymidine labelled macromolecules towards DNA digestion. Therefore we investigated the differences among the male morphotypes in their ability to synthesize DNA in organ culture.

Radiolabelled thymidine incorporation into the gonad during a 24-hour period and the amount of sperm released by the gonad into the culture media were determined (Fig. 1). For strong orange claw males, high levels of incorporation were associated with a low amount of sperm released by the gonad: 2970 cpm/10 mg tissue and 1.2×10^5 sperm/10 mg, respectively. On the other hand, high amounts of sperm were released, although very low levels of thymidine incorporation were detected in gonads of blue claw males (2.6×10^6 sperm/10 mg and 317 cpm/10 mg). The differences in sperm release and in the levels of [^{3}H]-thymidine incorporation between strong orange claw and blue claw males are statistically significant ($P < 0.005$ and $P < 0.05$, respectively).

Gonads removed from small males were characterized by a relatively high level of [^{3}H]-thymidine incorporation (4374 cpm/10 mg). This level of incorporation was significantly higher than that of blue claw males ($P < 0.01$), but not significantly different from the high levels of incorporation for strong orange claw and weak orange claw males. Gonads of small males released a relatively high amount of sperm into the media (1.5×10^6 sperm/10 mg). This was significantly higher than sperm released from strong orange claw and pretransforming strong orange claw male testes ($P < 0.01$), but not significantly different from the amounts released by blue claw males (Fig. 1).

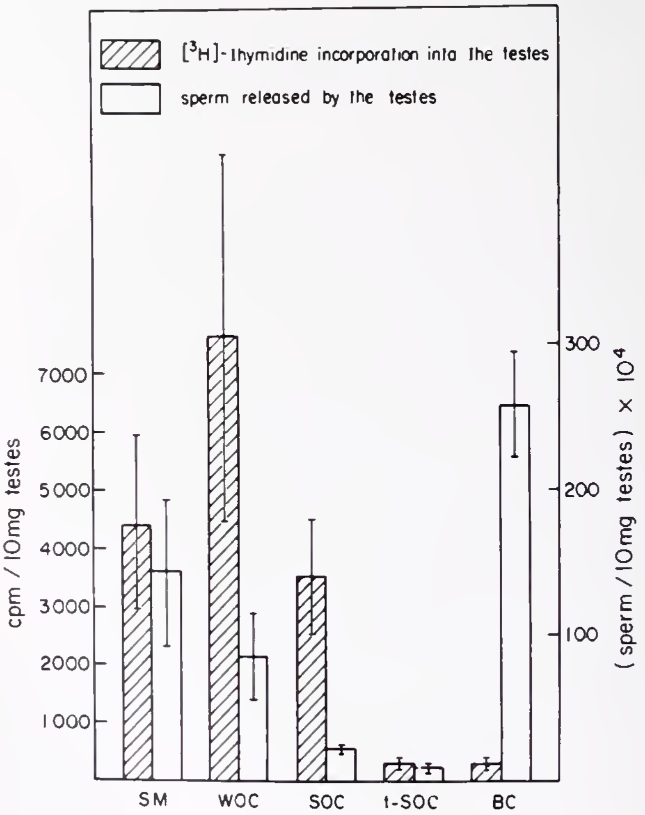

Figure 1. Sperm release and [^{3}H]-thymidine incorporation in organ culture of testes of *Macrobrachium rosenbergii* male morphotypes. The data represent means of 15 experiments and the variation is expressed as SE. SM = small male, WOC = weak orange claw, SOC = strong orange claw, t-SOC = pretransforming strong orange claw, BC = blue claw.

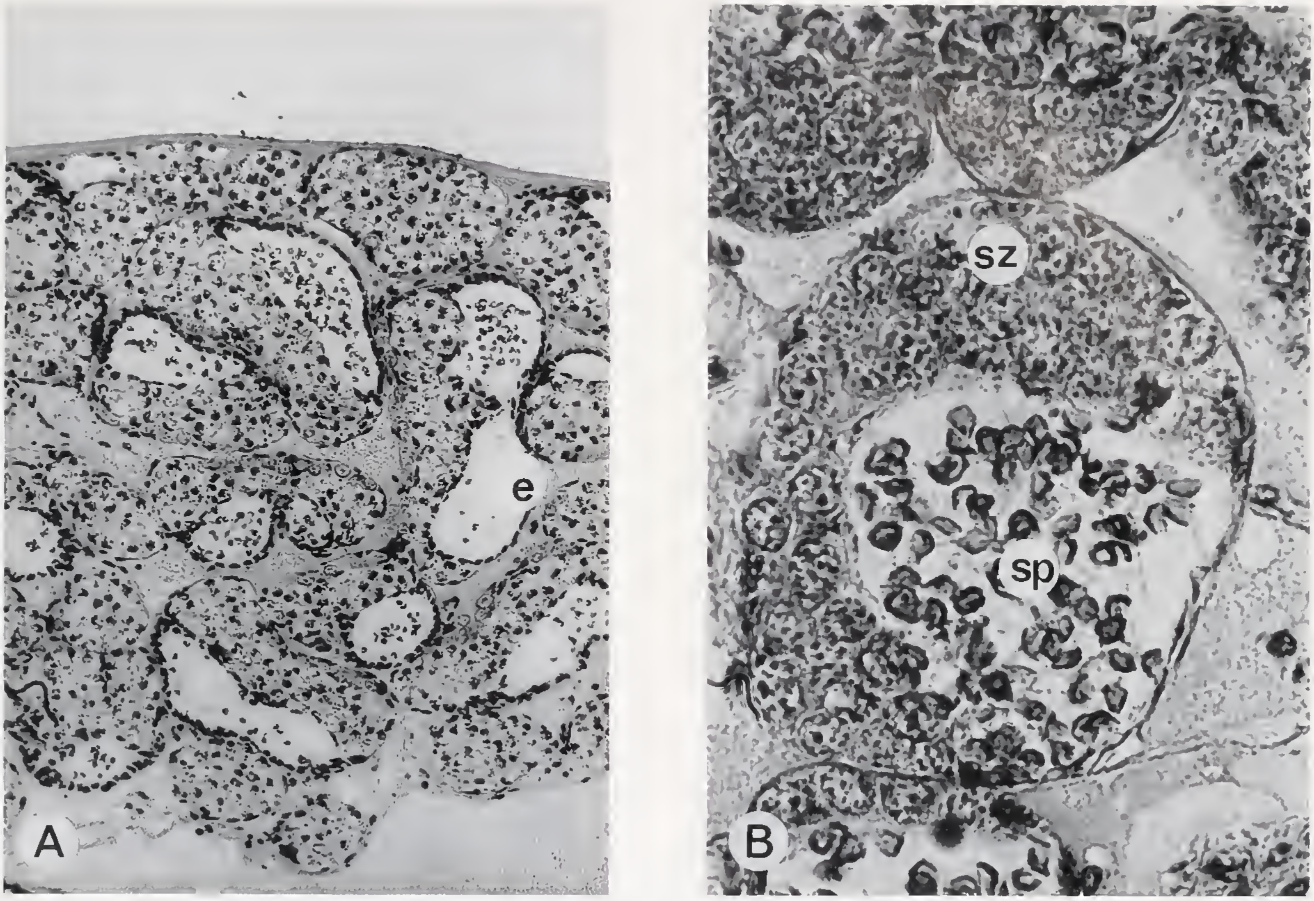

Figure 2. Light photomicrography of a cross-section through one testicular lobe removed from a small male: A. general cross-section (×50), B. a typical testicular cylinder (×200). e—epithelium, sz—spermatogenic zone, sp—mature spermatozoa.

The gonads removed from the pretransforming strong orange claw males were characterized by low levels of [^{3}H]-thymidine incorporation (331 cpm/10 mg). This level of incorporation is lower than all forms of males ($P < 0.01$) except blue claw males. The inactive testes of the pretransforming orange claw males were associated with a low amount of sperm released into the media, 9×10^4 sperm/10 mg, which was significantly low relative to other male morphotypes ($P < 0.01$), but not significantly different from the strong orange claw males.

Histological observations

The testicular lobes are composed of long cylinders compactly held together by connective tissue. Light microscopy revealed differences in the content of the cylinders among the different male morphotypes.

The testes of small males and weak orange claw males contained cylinders, most of which were enveloped by a single layer of epithelium (Fig. 2A, e). Part of the epithelium was multilayered and included cells of variable size, forming a spermatogenic zone (Fig. 2B, sz) containing germinal cells and sustentacular cells. Mature spermatozoa were seen in the lumen of each cylinder (Fig. 2B, sp).

The spermatogenic zone in the testes removed from strong orange claw and pretransforming strong orange claw males was thinner, occupying less space than in the testes of small males and weak orange claw males (Fig. 3A, sz). The cylinders contained spermatocytes (Fig. 3A, sc) which appeared similar in size, shape, and cytological features. While each cylinder contained spermatocytes of a uniform appearance, this appearance may vary from cylinder to cylinder (Fig. 3B, 1 and 2), probably corresponding to spermatogenic stage.

The testicular cylinder of blue claw males contained mature spermatozoa almost to the exclusion of other cell types (Fig. 4, sp). The spermatogenic zone was bearly observable (Fig. 4B, sz).

In all cases, the histological evidence for sperm accumulation in the dissected testes corresponded to the

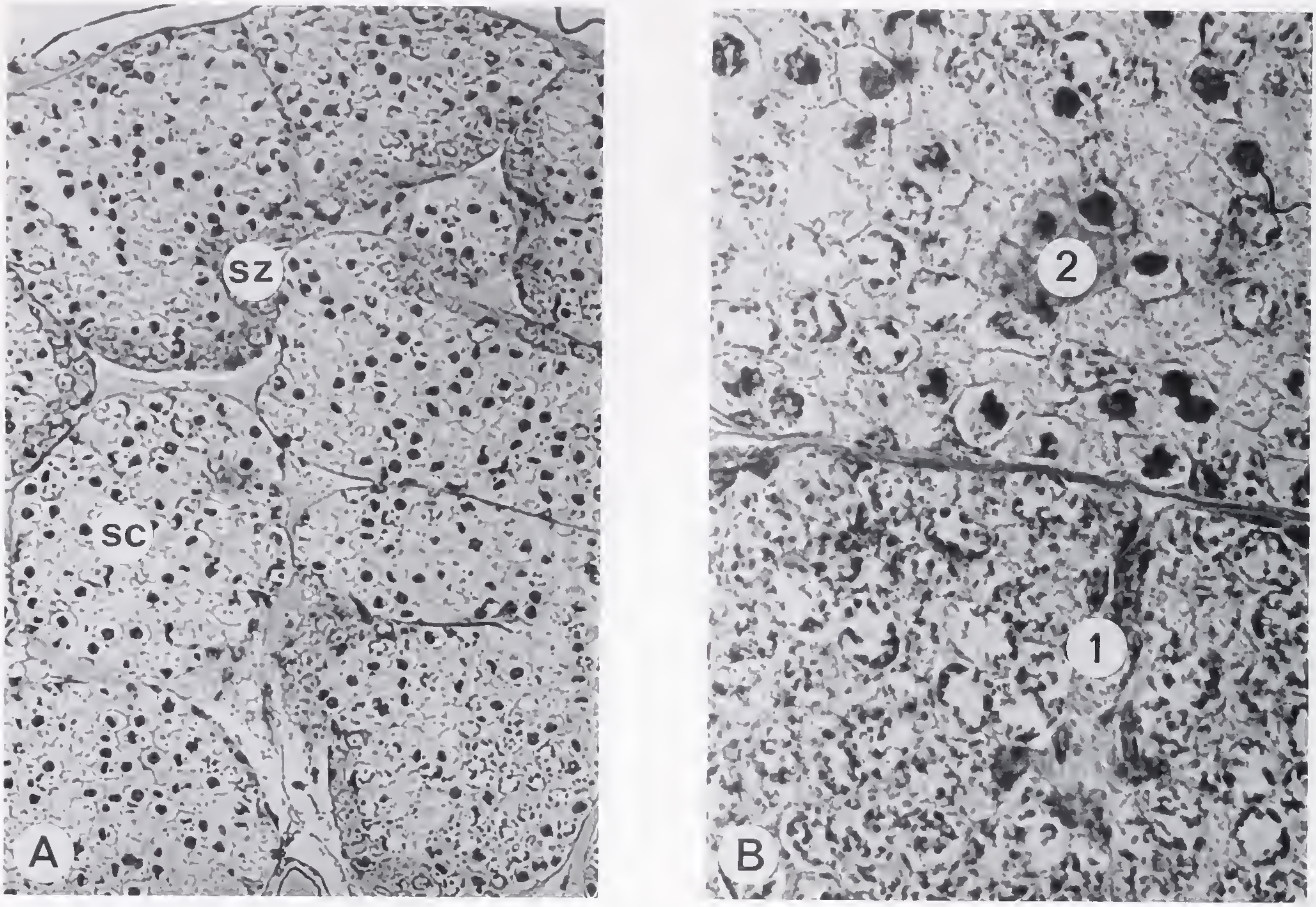

Figure 3. Light photomicrography of a cross-section through a testicular lobe removed from a strong orange claw male: A. general cross-section (×100), B. two neighboring testicular cylinders (×200) differing from each other in spermatogenic stage (1, 2). sz—spermatogenic zone, sc—spermatocytes.

amount of mature sperm released into the culture media after 24 hours of incubation.

Discussion

When polymorphism occurs among mature males in a population, different male morphotypes may adopt alternative mating strategies. This has been reported for insects (Alcock *et al.*, 1977; Ward, 1983), freshwater fishes (Keenleyside, 1972; Constantz, 1975; Gross and Charnov, 1980), some terrestrial mammals (Gadgil, 1972), and in crustaceans (Ra'anan and Sagi, 1985; Shuster, 1987). In most cases, individuals practice only a single reproductive strategy throughout their lifetimes. However, in some cases alternative mating patterns are part of a developmental sequence during a single lifetime (Dominey, 1980). The latter mechanism was described for sexually mature *Macrobrachium rosenbergii* males, in which small males may transform through the orange claw phase into the dominant blue claw males (Ra'anan and Sagi, 1985).

Among *M. rosenbergii* mature males, the small males employ a 'sneak copulation' strategy (Teleckey, 1984). Yet they retain the potential for somatic growth. Like small males in some fish species (Warner and Robertson, 1978; Robertson and Warner, 1978; Warner and Lejuene, 1985), the small *M. rosenbergii* male has a relatively large reproductive system (Sagi and Ra'anan, 1988) and relatively heavy gonads. These properties suit the small male mating strategy well, *i.e.*, numerous mating attempts and relatively little reproductive success (Ra'anan and Sagi, 1985). Hence, its testes contain relatively large amounts of mature sperm (Figs. 1, 2) and are actively engaged in spermatogenesis.

When the small male becomes an orange claw male, the balance between reproductive effort and somatic growth shifts. This is shown by an increase in growth rate (Ra'anan, 1982), an increase in the relative weight of the midgut gland, and a reduction in the relative weight of the reproductive system (Sagi and Ra'anan, 1988). As expected, these phenomena are accompanied by a de-

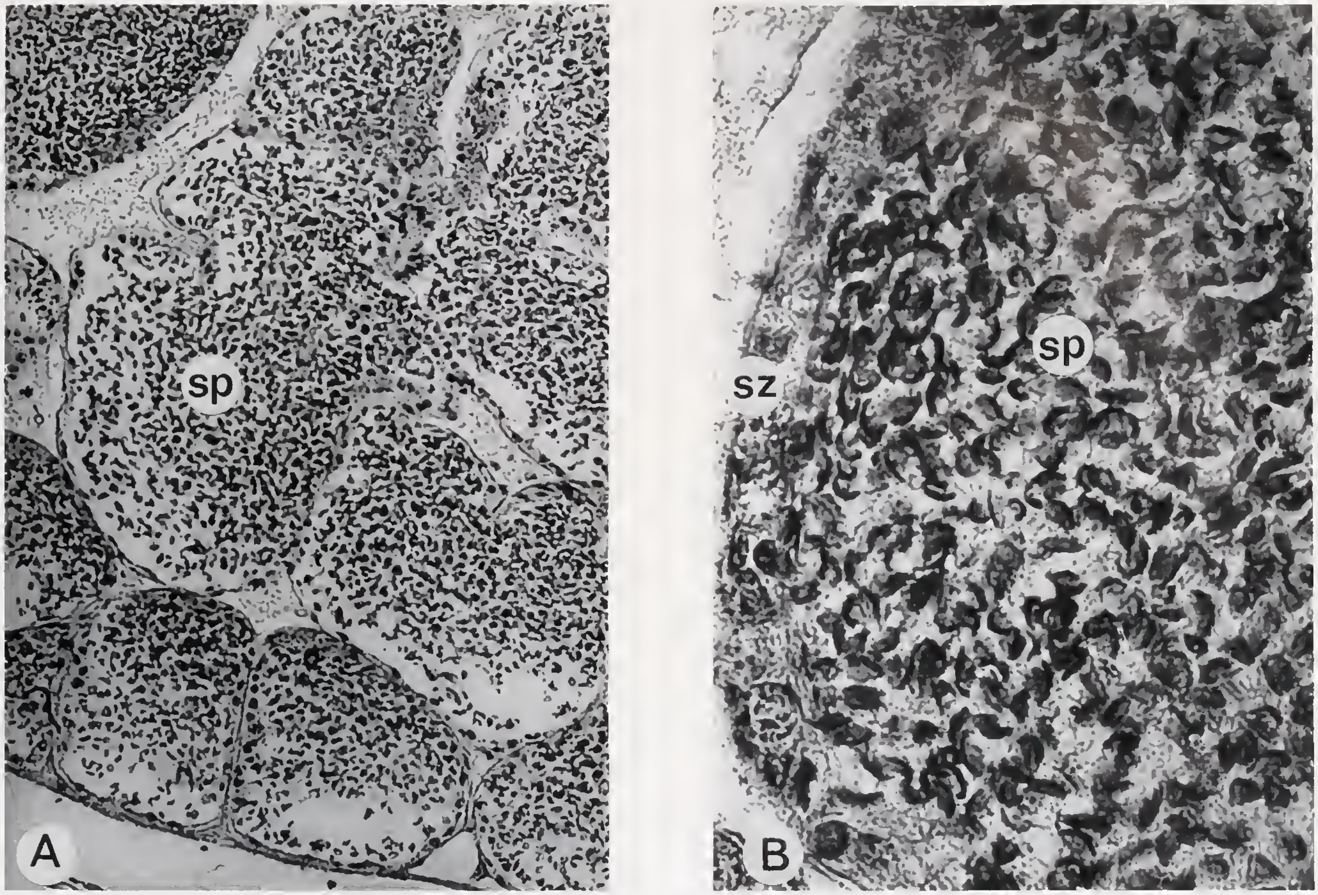

Figure 4. Light photomicrography of a cross-section of a testicular lobe removed from a blue claw male: A. general cross-section (×50), B. a typical testicular cylinder (×200). sp—mature spermatozoa, sz—spermatogenic zone.

crease in the relative weight of the gonad. Two parallel processes occur in the testes during the orange claw phase which indicate the intermediate nature of this morphotype. First, mature sperm, found in large amounts in the small male testes, are in reduced abundance in the weak orange claw males and almost disappear in the later strong and pretransforming orange claw stages. Thus, the orange claw male is almost sexually inactive, growing rapidly to reach dominant male size and appearance. Second, the spermatogenesis rate increases when small males molt to the weak orange claw male phase. Histologically, the [^{3}H]-thymidine incorporation data are supported by the multilayered spermatogenic zones within the testicular cylinders. In more advanced orange claw male phases, the spermatogenic activity decreases and almost ceases prior to metamorphosis into the blue claw male morphotype (Fig. 1). In the strong and pretransforming orange claw males, the spermatogenic zone becomes thinner while testicular cylinders become filled with spermatocytes (Fig. 3). Dougherty and Sandifer (1984) observed two apparently exceptional specimens that appeared to be in synchrony with respect to spermatogenesis. Our results suggest that these were orange claw males. These properties are consistent with the status of the orange claw morphotype as an intermediate stage, leading to transformation into the dominant blue claw morphotype.

The testes of the blue claw male contain only mature sperm, while the spermatogenic zone virtually disappears and spermatogenesis comes to a standstill. Thus, blue claw male testes are used exclusively for sperm storage, a characteristic that complements the dominant reproductive status of the blue claw male (Ra'anan and Sagi, 1985). That the blue claw male uses the stored sperm rather than producing sperm is consistent with the suggested pattern of replacement of blue claw males by transforming orange claw males in *M. rosenbergii* populations. This pattern of growth and metamorphosis has been termed the 'leap frogging' pattern (Ra'anan and Cohen, 1985).

In summary, whereas the small male both produces and stores sperm, the orange claw male primarily produces spermatocytes and the blue claw male only stores and uses mature sperm. Hence we can postulate that the small male possesses relatively undifferentiated testes, having the potential to develop—when environmental and social situations permit—through the intermediate phase of the orange claw into a dominant blue claw male.

The observed changes in the male gonads, from an active sperm-producing organ into a mature sperm storage organ, call for a more detailed study of the ultrastructural and biochemical changes in the spermatogenic process occurring in intermediate phases, among the distinct morphotypes, which are not addressed in the present study.

The appearance of distinctive male types, differing in reproductive activity and somatic growth, calls for studies on the mechanism of control of morphotypic differentiation. *M. rosenbergii* could be a valuable model for the study of endocrine regulation of growth *versus* maturation in crustaceans. The organ culture system developed here may also be useful in future studies of the effect of endocrine factors on the activity of the reproductive system in male crustaceans.

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Post-Embryonic Development of the Horseshoe Crab[1]

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Abstract. Individuals of *Limulus polyphemus* and *Tachypleus tridentatus* reached, respectively, the fourteenth-instar and tenth-instar stages during our nine-year rearing experiment. Body sizes were measured using exuviae and body specimens. The results made their growth steps clear, and body sizes of the juveniles at further growth stages could be estimated. We conclude from these data that *L. polyphemus* males generally molt 16 times to reach maturity in the ninth year; females molt 17 times to reach maturity in the tenth year. Similarly, we conclude from the presumptive numbers of growth stages that *T. tridentatus* males generally molt 15 times to reach maturity in the thirteenth year; females molt 16 times to reach maturity in the fourteenth year. Although we have few data on growth stages of *Tachypleus gigas* and *Carcinoscorpius rotundicauda,* it is thought that *T. gigas* males molt 12 times to reach adulthood, females molt 13 times, and that *C. rotundicauda* reaches maturity after the thirteenth molt in both males and females.

Introduction

Few have studied the post-embryonic development of horseshoe crabs. This may be because a method of culturing juveniles had not been established and that, in *Tachypleus tridentatus,* first instars did not grow into second instars within a year of hatching. Recently, Brown and Clapper (1981) described the procedures for collecting gametes, culturing embryos and juveniles, and maintaining adults of *Limulus polyphemus.* Until that time, they grew *Limulus* juveniles to the fifth-instar stage. However, the exact number of molts in the post-embryonic development of horseshoe crabs is not known, and it is not clear how many years they take to mature and how long mature animals live.

According to Shuster, *Limulus polyphemus* takes 9 to 11 years (Shuster, 1960) and 19 or fewer molts (Shuster, 1954) to reach maturity. Based on field observation and measurement of exuvial sizes, Asano (1942) reported that *T. tridentatus* reached maturity in 15 to 16 years after 17 or 18 molts. Goto and Hattori (1929) suggested that *T. tridentatus* molts 12 to 13 times before maturity.

The best way to confirm these animals' growth history would be to follow individual horseshoe crabs from hatching to maturity in their natural habitat. However, this is difficult to do for ten or more years without interruption. The alternative is to rear hatched horseshoe crab juveniles to maturity in a biological laboratory. Thus, Sekiguchi began to rear *T. tridentatus* and *L. polyphemus* from artificially fertilized eggs. The number of first instars used at the starting point was 100 or more for both species. Unfortunately, the last individual of *T. tridentatus* died at the tenth-instar stage, and the last *L. polyphemus* died at the fourteenth-instar stage. The plan of rearing them to adulthood was therefore abandoned.

During the experiment, however, the animals left us many exuviae and body specimens, enabling us to compile data on growth stages.

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Materials and Methods

Japanese horseshoe crabs (*Tachypleus tridentatus*) were collected from Hakata Bay, Fukuoka, Japan. Southeast Asian horseshoe crabs (*Tachypleus gigas* and *Carcinoscorpius rotundicauda*) were collected in the vicinity of Bangsaen, Thailand. American horseshoe crabs (*Limulus polyphemus*) were supplied by the Marine Biological Laboratory, Department of Marine Resources, Woods Hole, Massachusetts.

Just after hatching, *Limulus* individuals were reared in glass bowls (~6 cm diameter, ~3 cm depth) containing seawater of 20, 25, 30, or 35‰ salinity at 30–35°C. Seawater was recycled daily during Sekiguchi's stay in North Carolina (at the Duke University Marine Laboratory). Each glass bowl held 20 individuals. In Japan, *Limulus* juveniles were reared individually after the fifth-instar stage in normal seawater (34–35‰ salinity) at room temperature.

The first-posthatched juveniles of the Asian horseshoe crabs were cultured in bowls (~ 8.5 cm diameter, ~ 4.5 cm depth) which held 20 individuals and contained normal seawater. Asian horseshoe crab juveniles were reared individually after the first-instar stage in normal seawater at room temperature.

Individual horseshoe crab juveniles were cultured in seawater containers adequately sized to hold animals at different growth stages. The smallest container was an individual compartment about 34 × 34 × 30 mm deep for *T. tridentatus* second instar (prosomal width ~ 8 mm, total length ~ 10 mm), and the largest one was about 36 × 25.5 × 10 cm deep for *Limulus* fourteenth instar (prosomal width ~ 8 cm, total length ~ 14 cm). A layer of sand was placed in each compartment and container to allow burrowing. Culture seawater was prepared by filtering normal seawater and was changed daily (re-filtered seawater was sometimes used). Seawater was not recirculated or aerated.

After the second-instar stage, juveniles were fed daily. For each feed they were placed in seawater containers with freshly hatched brine shrimps, chopped earthworms (Tubificidae), or chopped Japanese littlenecks (Veneridae) for about 60–90 minutes.

Body sizes were measured at eight parts of the first instars and at nine parts of animals at growth stages from the second instar to adult, as shown in Figure 1. The sizes of the first to third instars were measured with a micrometer under a stereomicroscope, while the sizes of animals older than the third-instar stage were measured with slide calipers.

Subadult horseshoe crabs are externally similar regardless of sex, except for the gonopores; but after the last molt, the males of the four species are distinguished from the females by the claspers of the second prosomal appendages. The females of *Tachypleus* are distinguishable from males by the three shortened marginal (or movable) spines (Sekiguchi and Nakamura, 1979). Using these characteristics we determined whether an animal had grown up or not. However, most females of *Limulus* and *Carcinoscorpius* were also considered to be adult animals by their production of mature eggs (which we used for developmental experiments).

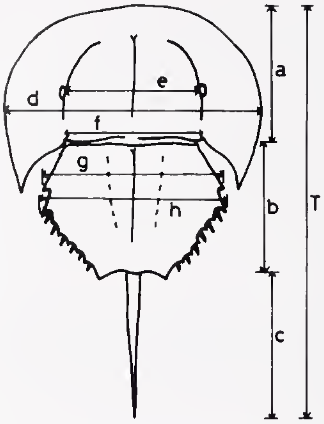

Figure 1. Horseshoe crab measurements. The parts whose lengths are measured are *a–h* and *T*. The first instar has no telson (*c*.).

Results

Early stages of Limulus *juvenile growth*

The first-instar stage. The lengths of the first-instar stage were 11–16 days at 20‰, 12–20 days at 25‰, 11–20 days at 30‰, and 13–24 days or more at 35‰ when 20 individuals were reared in a glass bowl. Their average values were 13.4, 15.3, 14.6, and 18.6 + α days (the last number, 18.6 + α, means that 17 individuals molted for the first time during the 24 days after hatching), respectively.

The second-instar stage. The second-instar stage lasted 10–12 (11.2) days at 20‰, 10–11 (10.3) days at 25‰, and 10–12 (10.9) days at 30‰ when the lengths were determined using the first 10 individuals grown to the third-instar stage (the average values are shown in parentheses). The growth of juveniles slowed greatly at 35‰.

The third-instar stage. The 12 third-instar juveniles molted for the third time 10–18 days after the second molt in seawater of 30‰ salinity at 30–35°C. The average length of this stage was 12.5 days.

The fourth-instar stage. Eleven of the above 12 fourth-instar juveniles became fifth-instar juveniles 13–20 days after the third molt; the average length of this stage was 15.8 days.

The fifth-instar stage. One of the two fifth-instar juveniles reached the sixth-instar stage 22 days after the fourth molt; the other reached it 23 days after the fourth molt.

After that, *L. polyphemus* juveniles were cultured in normal seawater (34–35‰) at room temperature. Many fifth-instar juveniles molted to become sixth-instar juveniles between September and mid-November, but no juveniles became seventh-instar juveniles within the first year.

The sixth-instar and later stages. The 23 sixth-instar juveniles of *Limulus* entered a second year, and 17 of them molted 3 times in the second year. The first sixth-molt in our experiment occurred in late April.

During the third year, *Limulus* juveniles molted twice, and the eleventh-instar juveniles entered the fourth year. They molted only once a year from the fourth year to the sixth year. This growth pattern was noted for *Limulus* juveniles whose posthatch development was successful. There were many exceptions: (1) the fifth-instar juveniles which molted four times in the first year molted four times in the second year. (2) Many normal sixth-instar juveniles molted four times in the second year and only once in the third year. (3) Many juveniles molted twice in the fourth year when they had molted only once in the third year.

Several *Limulus* juveniles were grown to the fourteenth-instar stage in the sixth year. They lived for another year or two without molting and then died.

Early stages of T. tridentatus *juvenile growth*

Freshly hatched juveniles of *T. tridentatus* never molted in the first year at room temperature. When they were reared at 30°C year-round, they molted only once late in the first year.

T. tridentatus juveniles molted for the first time in late June, for the second in mid-July, and for the third in mid-September of the second year. The fourth-instar juveniles passed the winter. In the third year, they molted for the fourth time between early June and early July and for the fifth time between early August and late September. Most of them molted once a year from the fourth year to the seventh year. The molts occurred between early June and late July in about 80% of animals in the fourth and fifth year.

Body sizes and relative growth

The mean values and standard deviations of body sizes of *L. polyphemus* from the first to fourteenth instars and of *T. tridentatus* from the first to the tenth instars are shown in Tables I and II, respectively.

The two relative growth patterns are presented by the allometric growth curves in Figure 2 using a logarithmic plot. (Their correlation coefficients were near 1.) The lin-

Table I

The means ± SD (standard deviations) of body sizes (mm) of Limulus polyphemus *juveniles continuously reared from the 1st-instar to 14th-instar stages*

	Instar stage													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
a	2.14 ± 0.30	3.06 ± 0.34	4.46 ± 0.24	5.70 ± 0.44	7.08 ± 0.57	8.80 ± 0.63	11.2 ± 0.83	13.9 ± 1.10	17.6 ± 1.79	23.0 ± 2.00	26.5 ± 3.82	31.0 ± 3.23	38.7 ± 5.34	48.0 ± 3.03
b	1.91 ± 0.25	2.12 ± 0.17	3.11 ± 0.21	4.06 ± 0.33	5.11 ± 0.46	6.41 ± 0.50	8.18 ± 0.59	10.4 ± 0.81	13.2 ± 1.20	16.8 ± 1.37	18.9 ± 2.16	22.5 ± 2.61	28.6 ± 3.86	35.6 ± 2.57
c		1.73 ± 0.19	3.44 ± 0.30	5.07 ± 0.60	7.04 ± 0.74	9.41 ± 0.96	12.8 ± 1.22	17.1 ± 1.66	23.0 ± 2.06	30.4 ± 3.65	35.5 ± 4.19	43.7 ± 6.84	59.7 ± 8.83	68.0 ± 3.00
d	3.30 ± 0.30	5.03 ± 0.20	7.03 ± 0.41	9.08 ± 0.87	11.3 ± 1.05	14.5 ± 1.18	18.6 ± 1.39	23.5 ± 1.78	29.5 ± 2.31	38.0 ± 2.93	43.7 ± 4.95	50.2 ± 7.30	64.4 ± 8.87	81.2 ± 5.27
e		2.90 ± 0.22	4.00 ± 0.20	5.03 ± 0.35	6.19 ± 0.46	7.60 ± 0.55	9.69 ± 0.75	12.2 ± 0.84	15.6 ± 1.30	19.8 ± 1.59	23.9 ± 2.03	28.3 ± 3.94	35.3 ± 4.20	44.0 ± 3.34
f	2.04 ± 0.20	2.98 ± 0.18	4.29 ± 0.27	5.41 ± 0.42	6.75 ± 0.57	8.46 ± 0.62	10.7 ± 0.79	13.6 ± 1.00	17.3 ± 1.43	21.6 ± 2.22	25.2 ± 2.17	29.5 ± 3.52	37.0 ± 4.20	45.8 ± 4.00
g		3.45 ± 0.18	4.83 ± 0.31	6.12 ± 0.54	7.62 ± 0.76	9.53 ± 0.77	12.1 ± 0.93	15.2 ± 1.16	19.0 ± 1.53	23.6 ± 1.70	27.4 ± 2.87	31.5 ± 3.99	39.8 ± 4.71	48.2 ± 4.35
h	2.87 ± 0.62	3.56 ± 0.20	4.91 ± 0.25	6.19 ± 0.49	7.73 ± 0.60	9.67 ± 0.65	12.3 ± 0.83	15.5 ± 1.16	19.3 ± 1.57	25.2 ± 2.40	27.7 ± 3.11	33.2 ± 3.90	41.7 ± 5.60	51.4 ± 4.27
T	4.05 ± 0.48	6.89 ± 0.59	11.0 ± 0.68	14.8 ± 1.21	19.1 ± 1.43	24.6 ± 1.74	32.1 ± 2.43	41.1 ± 3.25	54.3 ± 5.03	70.9 ± 6.46	80.0 ± 9.41	97.9 ± 10.9	127.8 ± 15.2	141.0
n	44	36	37	47	76	74	73	65	53	24	19	17	9	1

Body parts (*a*–*h* and *T*) measured are shown in Fig. 1.

The *n* shown in the lowest row represents a sample size for part *T*. It is the smallest in the sample sizes for parts *a* to *T* at each growth stage mainly because of imperfect telson exuviae (for example, sample sizes for *a* to *T* at stage 14 are 5, 5, 2, 5, 5, 5, 5, 5, and 1, respectively).

Table II

The means ± 1 SD (standard deviations) of body sizes (mm) of Tachypleus tridentatus *juveniles continuously reared from the 1st-instar to 10th-instar stages*

	Instar stage									
	1	2	3	4	5	6	7	8	9	10
a	3.32 ± 0.18	4.30 ± 0.41	5.76 ± 0.49	7.83 ± 0.31	10.2 ± 0.40	12.3 ± 0.58	15.7 ± 1.02	19.8 ± 1.24	23.6 ± 0.75	25.0
b	3.03 ± 0.17	3.06 ± 0.18	4.25 ± 0.17	5.58 ± 0.18	7.37 ± 0.25	9.18 ± 0.95	12.2 ± 0.94	15.8 ± 0.81	18.2 ± 0.33	21.1
c		2.60 ± 0.26	6.13 ± 0.46	9.93 ± 0.54	14.9 ± 0.92	21.1 ± 1.64	27.1 ± 2.73	38.0 ± 3.29	46.6 ± 0.70	53.1
d	5.98 ± 0.31	7.75 ± 0.36	10.7 ± 0.45	13.3 ± 0.53	17.5 ± 0.80	22.2 ± 1.44	28.0 ± 2.14	37.3 ± 2.92	42.7 ± 0.51	48.4
e	3.80 ± 0.14	4.49 ± 0.23	6.10 ± 0.26	7.82 ± 0.33	9.88 ± 0.48	12.6 ± 0.51	16.0 ± 1.05	20.3 ± 0.97	23.4 ± 0.91	26.6
f	3.89 ± 0.12	4.50 ± 0.35	6.20 ± 0.24	8.05 ± 0.34	10.3 ± 0.51	12.8 ± 0.62	16.2 ± 0.84	20.2 ± 1.07	23.6 ± 1.11	26.1
g	4.85 ± 0.17	6.00 ± 0.15	7.86 ± 0.28	9.96 ± 0.44	12.8 ± 0.61	16.1 ± 0.86	19.9 ± 1.22	26.1 ± 1.66	28.8 ± 0.90	32.1
h	4.82 ± 0.13	5.77 ± 0.23	7.78 ± 0.27	10.0 ± 0.36	13.0 ± 0.60	16.3 ± 0.92	20.6 ± 1.25	26.8 ± 1.73	30.0 ± 1.69	34.7
T	6.30 ± 0.19	9.95 ± 0.71	16.4 ± 0.77	23.3 ± 0.91	32.3 ± 1.19	42.6 ± 2.49	54.9 ± 4.25	74.4 ± 3.65	88.7 ± 0.40	99.2
n	33	22	12	13	13	21	24	9	2	1[1]

[1] All the sample sizes for *a* to *T* are 1.

Body parts (*a–h* and *T*) measured are shown in Fig. 1.

The sample size for *T* at each growth stage are shown in the lowest row (*n*). Generally it is smallest in the sample sizes for parts *a* to *T* (for example, sample sizes for *a* to *T* at stage 9 are 3, 3, 2, 3, 3, 3, 3, 3, and 2, respectively).

ear growth correlation means that the two parts are grown in proportion to each other, and a change in the slope means that a difference in growth rate between the two parts occurred. The growth curves other than that of the telson show only a straight line, suggesting that the growth correlation between prosoma width and the length of each of seven parts (except the telson) was invariable through post-embryonic development of *Limulus*. (Their relative growth coefficients are about 1 except for that of the total length.) On the other hand, the growth correlation between prosomal width and telson length changed twice: the curve showed the three phases. The first was the second through fourth instar, the second was the fourth through twelfth instar, and the third was the twelfth instar through adulthood (Fig. 2). The relative growth coefficients were 1.83, 1.23, and 1.03 for the first, second, and third phases, respectively.

In *T. tridentatus*, the allometric growth curves in a logarithmic plot showed a straight line except those for telson length and total length. The growth curves for the telson and total length consisted of three and two straight lines, respectively.

These states of growth were also characteristic of *T. gigas* and *C. rotundicauda*, although the body sizes of only five stages, from the first to fourth instars and the adult, were available for *T. gigas* and *C. rotundicauda*.

The estimation of growth stages

Stepwise growth after the fourteenth instar of *L. polyphemus* and the tenth instar of *T. tridentatus* could not be observed. However, it could be estimated using growth data from the first instar to the fourteenth instar (in *L. polyphemus*) or to the tenth instar (in *T. tridentatus*).

The rate of increase in the stepwise growth of each part is the most important information for estimating unknown animal sizes after the tenth-instar stage (*T. tridentatus*) and the fourteenth-instar stage (*L. polyphemus*). The rate of increase was 1.25 (*g* and *h*) to 1.37 (*c*) in *L. polyphemus*, and the average value was 1.28 for the nine parts (Table III). As shown in Figure 2, the points indicating adult and juvenile sizes were placed on an allomet-

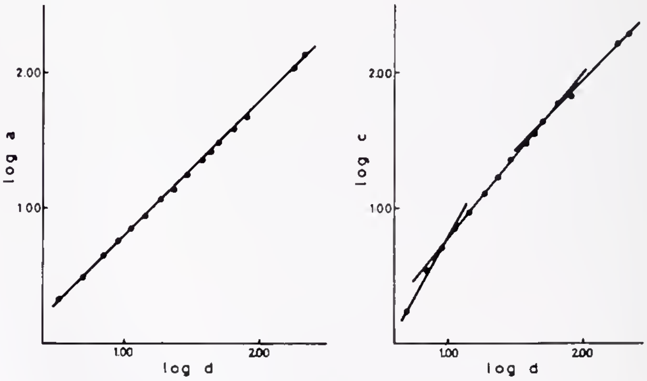

Figure 2. Relative growth in *Limulus polyphemus*. The allometric growth curve displays the logarithmic correlation between prosomal width (*d*) and prosomal length (*a*) or telson length (*c*). The uppermost and second uppermost dots on the lines present the mean sizes of females and males, respectively.

Table III

The presumed sizes (mm) of Limulus polyphemus *juveniles after the 14th-instar stage and means ± 1 SD (standard deviations) of adult sizes (mm) of* L. polyphemus

	Rate of increase	Instar stage					Adult	
		15	16	17	18	19	♂	♀
a	1.27	61	77	98	125	201	109.7 ± 7.0	138.5 ± 19.2
b	1.26	45	57	71	90	113	85.3 ± 4.6	106.3 ± 13.2
c	1.37	—	—	—	—	—	165.0 ± 20.2	194.5 ± 33.1
d	1.28	104	133	170	218	279	177.4 ± 11.4	213.9 ± 30.8
e	1.26	55	70	88	111	140	105.2 ± 7.5	133.6 ± 18.6
f	1.27	58	74	94	119	151	104.0 ± 6.9	130.0 ± 17.4
g	1.25	60	75	94	118	147	103.1 ± 6.5	129.1 ± 16.2
h	1.25	64	80	100	125	157	117.6 ± 7.3	148.8 ± 19.3
T	1.32	186	246	324	428	565	356.5 ± 31.6	437.9 ± 54.7

a–h and *T* are shown in Fig. 1.
The presumed sizes were calculated using the mean sizes of the 14th instars.
—, The size was not presumed.

ric growth line. Based on these results, the sizes of the respective parts after the fourteenth-instar stage were calculated using the appropriate rates of increase and mean sizes of the fourteenth instars (Table III). Similarly, the sizes after the thirteenth-instar stages of *T. tridentatus* were estimated and are shown with adult sizes in Table IV.

The presumptive numbers of growth (instar) stages of adult animals are summarized in Table V. From these data, we conclude that *Limulus polyphemus* males generally molt 16 times to reach maturity, and females, 17 times. Similarly, it is concluded from the presumptive numbers of growth stages (Table V) that *T. tridentatus* males generally molt 15 times to reach maturity, and females, 16 times.

We have few data on the growth stages of *T. gigas* and *C. rotundicauda*. However, it is thought that the stepwise growth of these two species progresses at almost the same rate as that of *L. polyphemus* or *T. tridentatus*, and thus their unknown body sizes after the fourth-instar stage were calculated (Tables VI, VII). According to these growth tables, *T. gigas* males molt 12 times to reach adulthood, and females, 13 times. *C. rotundicauda* reaches maturity after the thirteenth molt in both males

Table IV

The presumed sizes (mm) of Tachypleus tridentatus *juveniles after the 13th-instar stage and means ± 1 SD (standard deviations) of adult sizes (mm) of* T. tridentatus

	Rate of increase[1]	Instar stage					Adult	
		14	15	16	17	18	♂	♀
a	1.28	81	104	133	170	218	133.2 ± 12.6	166.1 ± 12.0
b	1.26	58	73	92	116	146	114.4 ± 12.9	136.6 ± 6.7
c	1.54	—	—	—	—	—	260.5 ± 31.7	298.8 ± 32.4
d	1.28	147	188	240	308	394	244.2 ± 18.7	278.4 ± 16.7
e	1.26	74	94	118	149	187	132.9 ± 7.0	164.4 ± 10.5
f	1.25	72	90	113	141	176	129.9 ± 8.1	157.6 ± 9.6
g	1.25	88	110	137	172	215	148.3 ± 10.2	177.4 ± 11.3
h	1.26	95	120	151	191	240	160.1 ± 11.1	188.5 ± 10.6
T	1.40	477	668	935	—	—	509.0 ± 49.9	600.4 ± 33.4

[1] Each mean rate of increase was determined using growth data from the 1st-instar to 9th-instar stages because of only one small 10th instar.
a–h and *T* are shown in Fig. 1.
The presumed sizes were calculated using the mean sizes of the 9th instars.
—, The size was not presumed.

Table V

The presumptive numbers of the growth (instar) stage of adults of Limulus polyphemus *and* Tachypleus tridentatus

		a	*b*	*d*	*e*	*f*	*g*	*h*
L. polyphemus	♀	18	19	18	19	18	18	19
	♂	17(~18)	18	17	18	17(~18)	17(~18)	(17~) 18
T. tridentatus	♀	17	18	(16~) 17	17 ~ 18	17 ~ 18	17	17
	♂	16	17	16	16 ~ 17	(16~) 17	16	16

a, b, d, e, f, g, and *h* are shown in Fig. 1.

and females. The rate of increase (1.26) used in the calculation was a mean rate of increase in *Limulus* body sizes except for the telson and total length.

Discussion

As the basis for estimating the total number of molts, it is necessary to know the relative growth of each part in *L. polyphemus* and *T. tridentatus.* Because the width of the prosoma (*d*) is easily measured and yields the smallest error in measurement and because Waterman (1954) and Shuster (1955) studied the relative growth of horseshoe crabs using the width of the prosoma, the relative growth between the width of the prosoma and each of eight lengths (*a, b, c, e, f, g, h,* and *T*) was examined. The growth of the *Limulus* telson was fastest in the first phase, and it preserved positive allometry in the second phase. Finally, the growth rate of the telson became similar to the widening of the prosoma. Because this tendency of telson growth was detected in *T. tridentatus,* the fact that the telson growth curve shows the three phases seems to be a remarkable characteristic of horseshoe crab growth in all species. However, positive allometry for the telson is predictable since the adult has a long telson but the first instar has none.

Waterman (1954) also indicated that the number of ommatidia in the horseshoe crab compound eye showed markedly rapid development in the initial growth phase and that telson elongation was marked by positive allometry during initial growth. The relative growth coefficients of six parts (not including the length of the telson and total length) are about 1 in both *L. polyphemus* and *T. tridentatus.* This means that each part except the telson enlarges at the same rate as does the width of the prosoma. Therefore, with the exception of the telsons, the shapes of the juveniles (except the first instar) and of mature adults are similar. Thus, we could estimate the juveniles' sizes after the tenth-instar (*T. tridentatus*) and

Table VI

The measured (stages 1 to 4) and presumed (stages 12 to 15) sizes (mm) of Tachypleus gigas *juveniles and means ± 1 SD (standard deviations) of* T. gigas *adult sizes (mm)*

	Instar stage				Rate of increase	Instar stage				Adult	
	1	2	3	4		12	13	14	15	♂	♀
a	4.08	6.26	8.19	10.1	1.26	64	81	102	128	86.8 ± 4.9	116.0 ± 8.8
b	3.19	4.90	6.25	8.00	1.26	51	64	81	102	74.1 ± 3.1	99.1 ± 5.6
c		3.92	7.81	12.9		—	—	—	—	171.0 ± 15.2	206.2 ± 19.5
d	8.33	12.1	14.8	19.2	1.26	122	154	194	244	163.0 ± 7.4	210.0 ± 15.6
e	4.51	5.57	7.01	9.27	1.26	59	74	93	118	80.1 ± 3.3	110.0 ± 6.4
f	4.53	6.04	7.66	9.73	1.26	62	78	98	124	82.4 ± 3.4	113.2 ± 14.8
g	5.63	7.94	9.80	12.2	1.26	78	98	123	155	89.9 ± 3.3	120.0 ± 6.9
h	6.23	9.20	10.8	13.5	1.26	86	108	136	172	107.5 ± 13.0	135.6 ± 7.4
T	—	—	—	—		—	—	—	—	331.6 ± 20.0	422.2 ± 29.1
n	10	10	10	7						23	30

a–h and *T* are shown in Fig. 1.
The sample sizes are shown in the lowest row (*n*).
The presumed sizes were calculated using the mean sizes of the 4th instars.
—, The size was not measured or presumed.

Table VII

The measured (stages 1 to 4) and presumed (stages 13 to 15) sizes (mm) of Carcinoscorpius rotundicauda *juveniles and means ± 1 SD (standard deviations) of* C. rotundicauda *adult sizes (mm)*

	Instar stage				Rate of increase	Instar stage			Adult	
	1	2	3	4		13	14	15	♂	♀
a	2.56	3.60	4.96	6.95	1.26	56	70	88	68.8 ± 3.6	74.1 ± 6.2
b	2.09	2.87	4.22	5.63	1.26	45	57	72	59.3 ± 3.0	62.4 ± 4.7
c		1.55	4.08	7.56		—	—	—	154.1 ± 9.1	160.6 ± 20.2
d	5.02	7.57	10.2	12.4	1.26	99	125	158	128.7 ± 3.7	133.8 ± 8.2
e	2.73	3.43	4.43	6.01	1.26	48	61	76	58.7 ± 2.2	63.7 ± 4.4
f	2.78	3.86	4.82	6.51	1.26	52	66	83	64.0 ± 2.5	68.5 ± 4.4
g	3.50	4.86	6.74	8.55	1.26	68	86	109	87.0 ± 2.6	89.8 ± 7.6
h	3.70	5.11	7.03	9.05	1.26	72	91	115	96.0 ± 3.3	99.6 ± 5.8
T	—	—	—	—		—	—	—	283.7 ± 11.0	296.6 ± 30.9
n	10	10	10	10					22	23

a–h and *T* are shown in Fig. 1.
The sample sizes are shown in the lowest row (*n*).
The presumed sizes were calculated using the mean sizes of the 4th instars.
—, The size was not measured or presumed.

fourteenth-instar (*L. polyphemus*) stages using the rate of increase in the stepwise growth of each part (Tables III, IV).

According to the growth table, we could determine the growth stages of two *Limulus* juveniles sent from Woods Hole. One individual estimated to be at the fourteenth-instar stage molted once and died. The rate of increase in this animal was 1.28 on average. Another individual was estimated to be at the seventeenth-instar stage. This animal also molted once; the rate of increase was 1.16. These facts strongly indicate that the rate of increase obtained from juveniles in the culture experiments is almost the same as that from the animals in their natural habitat.

However, there were some small mature males among the *L. polyphemus* specimens sent from Woods Hole in 1982. The total length of the smallest one was 229 mm and its prosomal width was 114 mm, suggesting that the sizes of these small males were similar to those of the presumptive sixteenth-instar juveniles. We cannot say whether they became adults after the fifteenth molt or became small-sized adults after the sixteenth molt because of the small rate of increase. According to Shuster (1982), in a local population (Plum Island Sound, Massachusetts) composed of a small type of *Limulus*, the mean prosomal width for males was 118 mm, and for females, 158 mm. It seems that *Limulus* in this population probably molted one fewer times than other *Limulus* to reach maturity—that is, 15 times in males and 16 times in females (Table III). On the other hand, the largest adults were found in the population at Bird Shoal, North Carolina, with a mean prosomal width of 232 mm for males and 327 mm for females. According to the growth table (Table III), these largest *Limulus* seem to have molted 17 times in males and 19 times in females. Thus, it is suggested that some *Limulus* reach maturity after one fewer or one more molt than normal animals, which molt 16 times in males and 17 times in females.

As in *Limulus*, the largest and the smallest *T. tridentatus* seemed to molt one more and one fewer times to reach maturity than normal animals in both females and males. When these results are compared with those of Goto and Hattori (1929), there are some obvious discrepancies between the two during stepwise growth. Although Goto and Hattori (1929) measured the body size of 1477 individuals (including 181 exuviae) of *T. tridentatus* from the first-instar stage to adulthood, their results were quite different from ours. However, they were farsighted in suggesting that females reached maturity after one more molt than males.

When we estimated the unknown body sizes of juveniles in *T. gigas* and *C. rotundicauda*, the growth rate used, 1.26, was a mean rate of increase in *Limulus* body sizes except for the telson and total length. This value is coincident with the mean values (1.26) calculated using the prosoma lengths (*a*) and opisthosoma widths (*h*) of five sets of Chinese *T. tridentatus* before and after a given molt (Chou and Cheng, 1950). According to the growth table (Table IV), *T. tridentatus* of the five sets molted for the fifth time (two sets), sixth time, seventh time, and eighth time. Chou and Cheng (1950) reported that the rates of increase in four other parts were 1.32 (*b*), 1.3 (*c*), 1.21 (*d*), and 1.21 (*T*).

Based on our estimation of the growth stages of horseshoe crab juveniles, there is a one-step difference in the instar stage between males and females of *L. polyphemus, T. tridentatus,* and *T. gigas.* There is no difference in the instar stage between males and females of *C. rotundicauda.* If the discrepancy is real, it is very interesting.

The horseshoe crabs lay their eggs in the sand near the high-tide level during the spring tide and there the eggs develop prior to hatching. The salinity of seawater was 18–33‰ on the Tatara coast, Saga, Japan, where *T. tridentatus* lays eggs (Sugita *et al.,* 1985). In Barnstable Harbor, on the north shore of Cape Cod, Massachusetts, where nests of *Limulus* eggs were found, the salinity was 20–32‰ (Robertson, 1970). Therefore, horseshoe crab embryos and hatched larvae and juveniles were expected to be tolerant of a wide range of environmental salinity levels. When the embryos were reared in seawater of 20–30‰, the developmental time until hatching was almost the same (Jegla and Costlow, 1982; Sugita *et al.,* 1985). When the post-hatch larvae and juveniles were reared in seawater of 20–30‰, it seemed that the difference in the effect of salinity on their growth was not significant (Jegla and Costlow, 1982; Laughlin, 1983). However, the development and growth of horseshoe crabs were slightly delayed in seawater with 35‰ salinity.

In our rearing experiment, the significant effect of salinity on the growth of *Limulus* young juveniles was not detected in 20–30‰. However, growth was delayed in 35‰. Regardless, this delay in juvenile growth did not affect the total number of molts in the first year.

From our rearing experiment, it is clear that *L. polyphemus* juveniles reach the sixth-instar stage within a year of when they were laid. Jegla and Costlow (1982) reported that freshly hatched juveniles of *L. polyphemus* molted about six times during the first year and several additional times during the second. However, no *Limulus* juveniles molted six times in our culture experiment during the first year. If the sixth instars had molted one more time during the first year in our rearing experiment, the sixth molt would have taken place in November or December. According to our records of the progress of *Limulus* growth, however, the number of *Limulus* juveniles that molt during this season is extremely small, probably due to the decline in temperature. Thus, it is thought to be unusual for *Limulus* juveniles to molt six times in the first year unless they are reared in a thermostatically controlled room. If the *Limulus* juveniles had been reared continuously in North Carolina, they might have molted for the sixth time.

Barlow *et al.* (1982, 1986) reported that light and vision play an important role in *Limulus* mating and breeding behavior. For juvenile growth, however, it seemed that the influence of light was not serious, because there was no difference in substantial growth of *Limulus* juveniles between the culture experiment, in which juveniles were treated with a 13/11 light/dark regimen (Jegla and Costlow, 1982), and our culture experiment, in which the lighting conditions depended on room conditions.

T. tridentatus juveniles up to tenth-instar stage (presumed according to our growth table) dwell at the tideland and, when the land is uncovered by the diurnal fall of the tide, they emerge from muddy sand and take food in tide pools (Kawahara, 1984). Therefore, it appears that the frequency of feeding in our rearing experiment was fairly adequate to grow horseshoe crab juveniles.

When the post-embryonic development of *L. polyphemus* goes smoothly, the animals molt five times in the first year, three times in the second year, twice in the third year, and once in the fourth year, after which they become twelfth-instar juveniles. It seems that after the twelfth instar they molt once a year, although there is no strong evidence for this assumption. On the other hand, the presumed adult stages of *L. polyphemus* correspond to the seventeenth-instar stage for males and to the eighteenth-instar stage for females. Therefore, *L. polyphemus* males reach maturity in the ninth year and females in the tenth year, providing that they molt once a year after the fourth year.

Shuster (1982) reported that the molting and aging sequence in Atlantic coast populations of *Limulus* generally included stage I–V in the first year, VI and VII in the second year, VIII and IX in the third year, and then a single molt each year from then on, with males reaching maturity at stage XVI in the ninth year; females had at least one more stage (XVII), reaching maturity one year later than males. However, Shuster determined the growth stages and ages using eight exuviae derived from a female *Limulus* and many immature and adult animals collected in the field, so we cannot compare our data precisely with his data, although his conclusion is fairly close to ours.

In *T. tridentatus,* the first instars pass the winter and molt three times in the second year, twice in the third year, and once in the fourth year, reaching the seventh-instar stage. After this they molt once a year. The sizes of male and female adult *T. tridentatus* corresponded to those of the presumptive sixteenth- and seventeenth-instar juveniles, respectively. Thus, male *T. tridentatus* are mature in the thirteenth year while females are mature in the fourteenth year.

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A Physiological Comparison of Bivalve Mollusc Cerebro-visceral Connectives With and Without Neurohemoglobin. III. Oxygen Demand

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Abstract. Several bivalve mollusc species possess neurohemoglobin in their nervous systems whereas most species do not. The cerebro-visceral connectives of *Tellina alternata* and *Spisula solidissima* with neurohemoglobin and *Tagelus plebeius* and *Geukensia demissa* without neurohemoglobin exhibit similar electrical characteristics dictated mostly by axon size (0.3–0.4 μm mean axon diameter, Kraus *et al.*, 1988). Action potential conduction is sensitive to a depletion of both ambient and neurohemoglobin-bound oxygen. Connectives without neurohemoglobin and connectives with carbon monoxide neurohemoglobin ceased to conduct action potentials within 5–10 minutes after exposure to anoxic conditions. Connectives with neurohemoglobin conducted action potentials throughout the time course of neurohemoglobin deoxygenation, lasting 20–30 minutes.

Connectives without neurohemoglobin exhibited an approximate five-fold elevation in oxygen consumption rate during action potential conduction, as predicted by axon diameter. However, connectives with neurohemoglobin consumed only ⅓ this amount of oxygen during electrical activity. The mechanism for this increased efficiency in action potential conduction is unknown, but the rate of oxygen consumption nearly matches the rate of neurohemoglobin oxygen unloading *in situ*. An operational aerobic nervous system might enable animals to maintain neuromuscular activity during hypoxic or anoxic conditions.

Introduction

Tissue hemoglobins supply oxygen to the surrounding tissue, and the ensuing aerobic metabolism produces energy which drives tissue function. In nervous tissue, the maintenance of ion gradients for action potential conduction requires energy to drive membrane-bound ionic pumps. Although the nervous systems of several organisms tolerant of anoxic conditions remain functional anaerobically (Mangum, 1980; Surkykke, 1983), total metabolic activity is sharply depressed (Ellington, 1981; Hochachka and Somero, 1984). In general, continued nervous activity is dependent on aerobic metabolism, as demonstrated in single axons (Maruhashi and Wright, 1967), compound nerves (Wright, 1946, 1947; Larrabee and Bronk, 1952), and brain cortex tissue slices (Bingmann *et al.*, 1984). For a review, see De Weer (1975).

Several species of bivalve molluscs possess substantial quantities of hemoglobin in their nervous systems, while most species do not. We have demonstrated that bivalve cerebro-visceral connectives with and without neurohemoglobin located in glial cells have similar electrical characteristics which are dictated by similar mean axon diameter (0.3–0.4 μm; Kraus *et al.*, 1988). In addition, both types of connectives possess comparable proportions of axon and glial cell volumes. However, axon bundles in cerebro-visceral connectives with neurohemoglobin are significantly smaller and contain fewer axons than in connectives without neurohemoglobin. This design provides more contact area and shorter mean distances between axons and glial cells. Thus, oxygen delivery from neurohemoglobin-containing glial cells to axons may be further enhanced (Kraus *et al.*, 1988).

Based on the oxygen affinity characteristics of the neurohemoglobin and the possibly low oxygen permeability of the perineural sheath, bivalve neurohemoglobin may

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operate as an oxygen store for aerobic metabolism during anoxic conditions (Doeller and Kraus, 1988). Initial studies indicated that during anoxic conditions, the neurohemoglobin-containing cerebro-visceral connectives of *Tellina alternata* were able to conduct action potentials until neurohemoglobin deoxygenation was complete (Kraus and Colacino, 1986). To determine how effective the oxygen store would be, we needed to show that neurohemoglobin-containing connectives (1) depend on oxygen for action potential conduction and (2) consume oxygen at a rate that reasonably matches the neurohemoglobin oxygen unloading rate. In this investigation, we determined the oxygen demand of bivalve cerebro-visceral connectives with and without neurohemoglobin and their ability to conduct axon potentials in anoxic conditions.

Materials and Methods

Animals

Entire cerebro-visceral connectives were obtained from three species of bivalve molluscs with neurohemoglobin, *Tellina alternata, Spisula solidissima* and *Macrocalista nimbosa,* and two species without neurohemoglobin, *Tagelus plebeius* and *Geukensia demissa.* Animal collection (except of *M. nimbosa* collected from sandy mud flats at Long Beach, North Carolina), laboratory maintenance and cerebro-visceral connective dissection have been described previously (Kraus *et al.*, 1988).

Oxygen consumption rates

Basal and active oxygen consumption rates of entire cerebro-visceral connectives were measured with a flow-through respirometer similar in design to that used to measure the oxygen consumption rate of crab nerves (*cf.* Fig. 1, Baker and Connelly, 1966). To load the respirometer, a cerebro-visceral connective was inserted into a fluid-filled glass capillary. Bathing seawater (35 parts per thousand salinity) was drawn (Harvard syringe pump) from a reservoir into one end of the capillary and past the connective. The seawater PO_2, lowered by connective respiration, was detected by a polarographic oxygen sensor (Diamond Electrotec) at the other end of the capillary. The respirometer was housed within a temperature-controlled water jacket. All experiments were conducted at 15 ± 0.5°C for *S. solidissima* and 20 ± 0.5°C for the other species. The reservoir seawater was sterilized by filtration (0.45 μm, Millipore) or by near-boiling (approximately 90°C) in a microwave oven and equilibrated with air or air plus 3–5 mmHg PCO through an aeration port. This CO tension is sufficient to saturate the cerebro-visceral connective neurohemoglobin and remove its oxygen binding ability (Doeller and Kraus, 1988). The mass of the connective segment inside the capillary, responsible for the decline in oxygen tension, was computed as the product of the total mass of the connective and the ratio of the length of the connective in the capillary to its total length.

Capillaries of different bores were used to accommodate connectives of different diameters to minimize the fluid volume surrounding the connective. The flow rate through the capillary, which depended on connective and capillary diameters, was adjusted to reduce both the boundary layer at the oxygen sensor and the response time of the respirometer and at the same time to ensure an easily detectable drop in fluid oxygen tension. The sensor output was monitored continuously with a chart recorder or sampled at 5 Hz with an A/D-interfaced microcomputer (C.U. Electronics; OSI, Inc.). The microcomputer was programmed to calculate and record oxygen consumption rates graphically and numerically on a dot matrix printer (Epson) used as a chart recorder.

The initial oxygen consumption rate was often elevated, presumably due to the mechanical stimulation associated with loading the cerebro-visceral connective into the respirometer (see Fig. 1), but it usually stabilized within 20 min at a lower level denoted as the unstimulated or resting oxygen consumption rate. The connective was then stimulated at various pulse rates, 0.25–5.0 pulses per second, with supramaximal stimuli, approximately 5–10 V, at 1 ms duration. Stimulation was typically delivered for 5 min to allow seawater entering the top of the capillary at the onset of stimulation to pass over the entire connective while active. In this way, the declining oxygen tension of the seawater passing by the connective reached a relatively constant level before stimulation was terminated. The stable oxygen consumption rate at this time was denoted as the active rate. The connective was then allowed to resume its resting oxygen consumption rate before stimulation was repeated, which usually occurred within 0.5–1 h. Each connective was subjected to 4–8 stimulation bouts. Selected connectives were subsequently placed in a nerve-chamber gas-slide (*cf.* Fig. 1, Kraus *et al.*, 1988) to demonstrate continued electrical excitability after an experiment.

Action potential conduction

Individual anterior segments (25–30 mm long) of connectives with and without neurohemoglobin were suspended across a series of 12 platinum electrodes in the temperature-controlled nerve-chamber gas-slide. Stimulating electrodes were connected to a stimulator (Grass SD9) and recording electrodes were connected to a preamplifier (Narco) and microcomputer (Apple IIE) equipped with oscilloscope hardware and software (R.C.

Electronics). The slide was sealed with upper and lower glass lids and placed in the light path of a microspectrophotometer (Colacino and Kraus, 1984) in order to monitor the fractional saturation of neurohemoglobin (Doeller and Kraus, 1988). Gas ports connected the nerve-chamber to a gas delivery system. Connectives were thus exposed to different gas tensions while action potential conduction and, when present, neurohemoglobin fractional saturation were monitored simultaneously. The compound action potentials of cerebro-visceral connectives exhibit a predominant slow velocity spike associated with the vast majority of small diameter axons within each connective (see Fig. 6, Kraus *et al.*, 1988). The amplitude of this spike was used as the measure of action potential conduction.

During a stimulation bout, a connective segment was first exposed to flowing humidified air (100 ml/min). Action potential conduction was monitored and when neurohemoglobin was present, an absorption spectrum was recorded. After at least 15 minutes, the flowing gas was switched to humidified 99.999% N_2 and neurohemoglobin deoxygenation and action potential conduction were monitored. (Gas washout required only a few seconds because the volume of nerve-chamber plus input line was less than 1 ml.) When deoxygenation was complete and/or action potential conduction ceased, the connective was re-exposed to humidied air. After rapid neurohemoglobin reoxygenation and the return of electrical excitability, usually within 15 min, the experiment was repeated. During experiments with 3–5 mmHg PCO present, a carbon monoxide neurohemoglobin spectrum was recorded before switching to N_2.

Ouabain was used in several experiments to inhibit the Na-K pumps during action potential conduction. After electrical activity was demonstrated, a drop of filtered seawater with 1 m*M* ouabain was placed on a connective between the stimulating and recording electrodes. At the end of a 30 min equilibration period, the connective was stimulated at 1 pulse per second with 5–10 V, 1 ms duration, and compound action potential amplitude was recorded.

Results

Oxygen consumption rates

Resting and active oxygen consumption rates of bivalve cerebro-visceral connectives with and without neurohemoglobin are shown in Figure 1. The short delays seen between the start of stimulation and initial increase in the consumption rate and between the end of stimulation and initial decrease in the consumption rate may be attributable to the transit time of the slowly flowing seawater past the connective to the oxygen sensor. The differences in resting oxygen consumption rates were not significant, but connectives without neurohemoglobin appeared to consume oxygen at a slightly higher rate than connectives with neurohemoglobin ($0.1 < P < 0.25$, hierarchical ANOVA; Fig. 1, Table I). Inherent or spontaneous low-level electrical activity in the unstimulated connectives could contribute to the resting oxygen consumption rates.

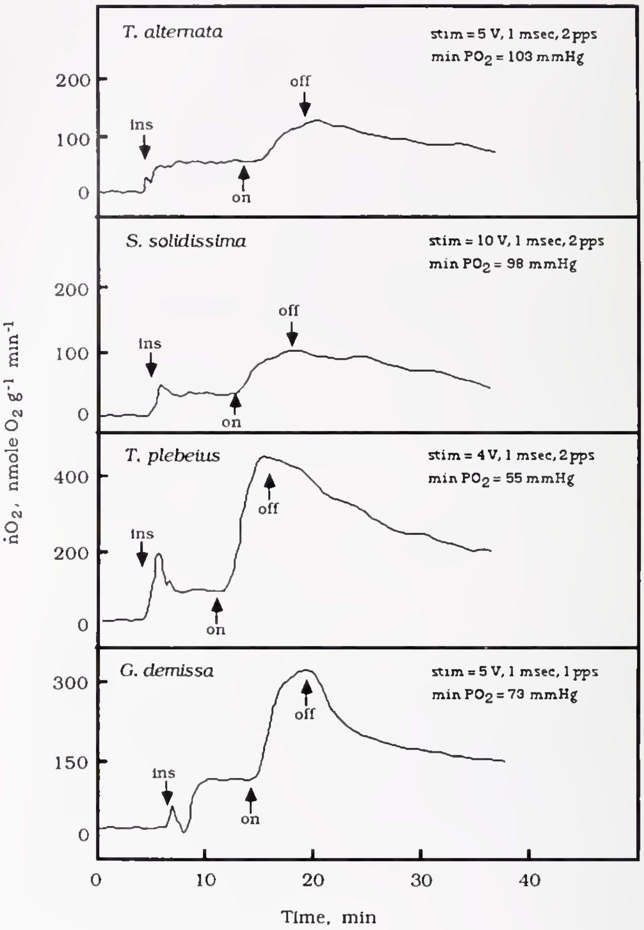

Figure 1. The resting and active oxygen consumption rates of bivalve cerebro-visceral connectives. The arrows refer to the times at which each connective is inserted into the respirometer (ins), the stimulation begins (on) and ends (off). The stimulation voltage, duration, and frequency and the minimum PO_2 are presented in the upper right hand corner of each trace.

Upon stimulation, connectives with and without neurohemoglobin all displayed an increase in oxygen consumption rate (Fig. 1, Table I). Because externally recorded compound action potentials displayed similar amplitudes in all preparations, the applied supramaximal stimulus was assumed to recruit as many axons as were excitable. Even so, the oxygen consumption rates of connectives without neurohemoglobin increased to a

Table I

Oxygen consumption rates of bivalve cerebro-visceral connectives

Pulses per second	Units	*Tellina alternata*	*Spisula solidissima*	*Macrocallista nimbosa*	*Tagelus plebeius*[a]	*Geukensia demissa*[a]
0	nmol O_2 g^{-1} min^{-1}	49.4 ± 16.1 (11)[b]	65.9 ± 19.6 (8)	48.8 (2)	83.0 ± 38.5 (15)	105.0 ± 46.8 (7)
2	nmol O_2 g^{-1} min^{-1}	101.4 ± 40.0 (5)	96.2 ± 14.3 (7)	126.0 (2)	505.2 ± 43.3 (5)	395.7 ± 67.1 (4)
2	nmol O_2 g^{-1} $pulse^{-1}$	0.84	0.80	1.05	4.21	3.30
2	nmol O_2 g^{-1} $pulse^{-1}$ above resting	0.43	0.25	0.64	3.52	2.42

[a] Species without neurohemoglobin.

[b] Numbers are given as average ± standard deviation (number of repetitions).

much greater extent than the rates of connectives with neurohemoglobin (Fig. 1, Table I). During experiments with neurohemoglobin-containing connectives, minimum PO_2 of the air-equilibrated seawater drawn through the capillary rarely fell below 100 mmHg, a magnitude greater PO_2 than required to fully saturate the neurohemoglobin *in situ* (Doeller and Kraus, 1988). In addition, the active oxygen consumption rates remained the same when the neurohemoglobin oxygen binding ability was eliminated with 3–5 mmHg PCO ($0.1 < P < 0.375$). The resting and active oxygen consumption rates of connectives without neurohemoglobin were not affected by this partial pressure of carbon monoxide ($0.1 < P < 0.375$).

The oxygen consumption rates of connectives with and without neurohemoglobin generally increased with increasing stimulus frequency (Fig. 2). Connectives without neurohemoglobin exhibited maximum oxygen consumption rates at a stimulus frequency of 2–3 pulses per second (Fig. 2). At higher stimulus rates, fewer axons may be conducting action potentials, as evidenced by a decrease in compound action potential amplitude, and this may lead to the observed decline in oxygen consumption rates. In contrast, connectives with neurohemoglobin exhibited nearly constant oxygen consumption rates from 0.5 to 5 pulses per second (Fig. 2). In an attempt to increase the consumption rates, stimulus strengths up to 50 V at 10 pulses per second were delivered, but action potential amplitude rapidly dropped as the connectives fatiqued and oxygen consumption rates actually declined.

Action potential conduction

Cerebro-visceral connectives with and without neurohemoglobin conducted compound action potentials at stimulus frequencies of 1–2 pulses per second for more than one hour under a flow of humidified room air, with less than a 10% change in amplitude or conduction velocity. At the onset of 99.999% N_2, compound action potential amplitude in connectives without neurohemoglobin rapidly declined and became indistinguishable from baseline in 4–8 minutes (Table II; Fig. 3). In contrast, action potential amplitude in connectives with neurohemoglobin remained elevated for about 20–30 min, or until the neurohemoglobin was nearly deoxygenated (Table II; companion neurohemoglobin deoxygenation traces lasted 18–26 minutes, see Fig. 3, Doeller and Kraus, 1988). When more than 90% of the neurohemoglobin oxygen store was effectively displaced by 3–5 mmHg PCO prior to N_2 exposure, action potential amplitude reached baseline in 5–10 minutes after N_2 exposure (Table II; Fig. 3). In air-equibrated conditions when ouabain was applied to directly inhibit Na-K pumps during stimulation, action potential amplitude of connec-

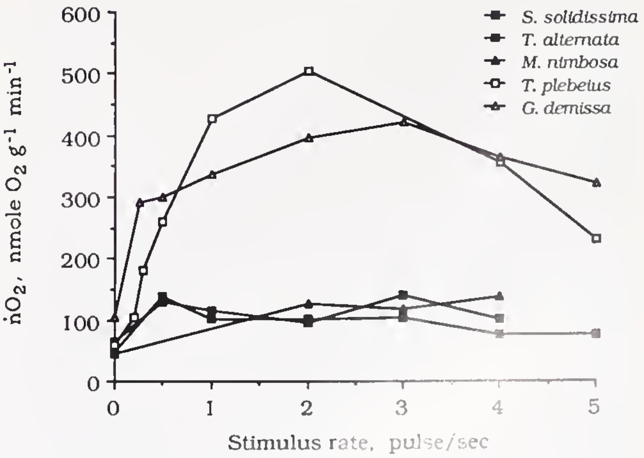

Figure 2. The effect of stimulus rate on the oxygen consumption rates of bivalve cerebro-visceral connectives. The closed symbols refer to connectives with neurohemoglobin, the open symbols refer to connectives without neurohemoglobin.

Table II

Time in minutes required for compound action potential amplitude of bivalve cerebro-visceral connectives to reach 50% of starting value after change in condition

Conditions	*Tellina alternata*	*Spisula solidissima*	*Tagelus plebeius*[a]	*Geukensia demissa*[a]
Air to N_2[b]	24.7 ± 6.0 (7)[c]	18.0 ± 3.8 (6)	3.9 ± 1.4 (4)	4.4 ± 1.9 (4)
Air + CO to N_2[b]	6.2 ± 1.3 (5)	6.3 ± 3.3 (4)	3.5 ± 0.7 (3)	5.5 ± 1.2 (3)
Air to air + 1 m*M* ouabain[d]	1.7 ± 0.8 (3)	2.5 ± 1.0 (3)	1.6 ± 1.3 (3)	2.3 ± 0.3 (3)

[a] Species without neurohemoglobin.

[b] Stimulus frequency 2 pulses per second.

[c] Numbers are given as average ± standard deviation (number of repetitions).

[d] After 30 min equilibration; stimulus frequency 1 pulse per second.

tives both with and without neurohemoglobin approached baseline in 5 minutes or less and electrical excitability was eliminated in the region of application of the drug (Table II).

Discussion

The basal oxygen consumption rate of any tissue represents the metabolic cost of cellular maintenance, and is characteristic of that tissue. In accordance, the mass specific resting oxygen consumption rates of nonmyelinated nerves do not vary greatly from one nerve type to another (Fig. 4; Ritchie, 1973). On the other hand, action potential conduction is a membrane phenomenon and the commensurate increase in oxygen consumption rate is a function of total axonal surface area (Ritchie, 1973). Compound nerves with small diameter axons contain more membrane surface area and possibly more energy-consuming Na-K pumps per gram than nerves with large diameter axons (Ritchie, 1973). Moreover, small diameter axons have a large surface area to volume ratio, and the dependency on active membrane-bound ionic pumps to maintain ion gradients may be high to compensate for the large fraction of ion content exchanged per action potential. Nonmyelinated bivalve cerebro-visceral connectives with and without neurohemoglobin, possessing 0.3–0.4 μm mean diameter axons, lose a calculated 0.05% of total cellular potassium per action potential (Kraus, 1986) whereas giant squid axons lose 0.000002% (Aidley, 1981). In experiments when Na-K pumps were inhibited by ouabain, connectives with and without neurohemoglobin lost electrical excitability in a short time, after conducting only a few hundred action potentials (Table II). Ouabain may interfere with the uptake of extracellular potassium by glial cells, as well as inhibit Na-K pumps (Johnston and Roots, 1972; Aidley, 1981). Thus, action potential conduction in bivalve connectives exhibits a strong dependancy on operational Na-K pumps which in turn rely on aerobic ATP production (Table II; see below). In fact, the magnitude of the increase in active oxygen consumption rate above resting is an inverse function of mean axon diameter, as shown in Figure 4. For example, the active oxygen consumption rate of giant squid axons is only a fraction greater than the resting rate (Connelly and Cranefield, 1953), but the active rate of small diameter garfish olfactory axons is many times greater than resting (Ritchie and Straub, 1979).

The resting and active oxygen consumption rates of bivalve cerebro-visceral connectives without neurohemoglobin are consistent with the trend displayed by other nonmyelinated nerves, based on axon diameter (lines c and e, Fig. 4). However, although the resting oxygen consumption rates of connectives with neurohemoglobin are similar to others, the active oxygen consumption rates are much lower than predicted by axon diameter (lines b, d, and f, Fig. 4) or total axonal surface area (Kraus *et al.*, 1988). Connectives with neurohemoglobin consume substantially less extra oxygen per impulse (2 pulses per second) than connectives without neurohemoglobin (Table I). Nonmyelinated garfish olfactory nerves, possessing 0.24 μm mean diameter axons and lacking neurohemoglobin, consume 2.46 nmol O_2 per g per impulse above resting (Ritchie and Straub, 1979), which is in close agreement with bivalve connectives without neurohemoglobin (Table I). The discordance in active oxygen consumption rates between connectives with and without neurohemoglobin was unexpected because both types of connectives exhibit very similar electrical and anatomical characteristics (Kraus *et al.*, 1988). Several possible explanations for this difference are proposed in the following discussion.

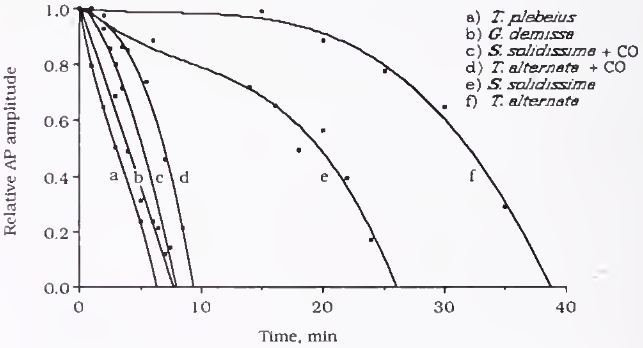

Figure 3. The relative action potential amplitude of bivalve cerebro-visceral connectives after the removal of oxygen at time 0, at 2 pulses per second. Lines a through d refer to connectives without neurohemoglobin or with an inactive neurohemoglobin, lines e and f refer to connectives with functional neurohemoglobin.

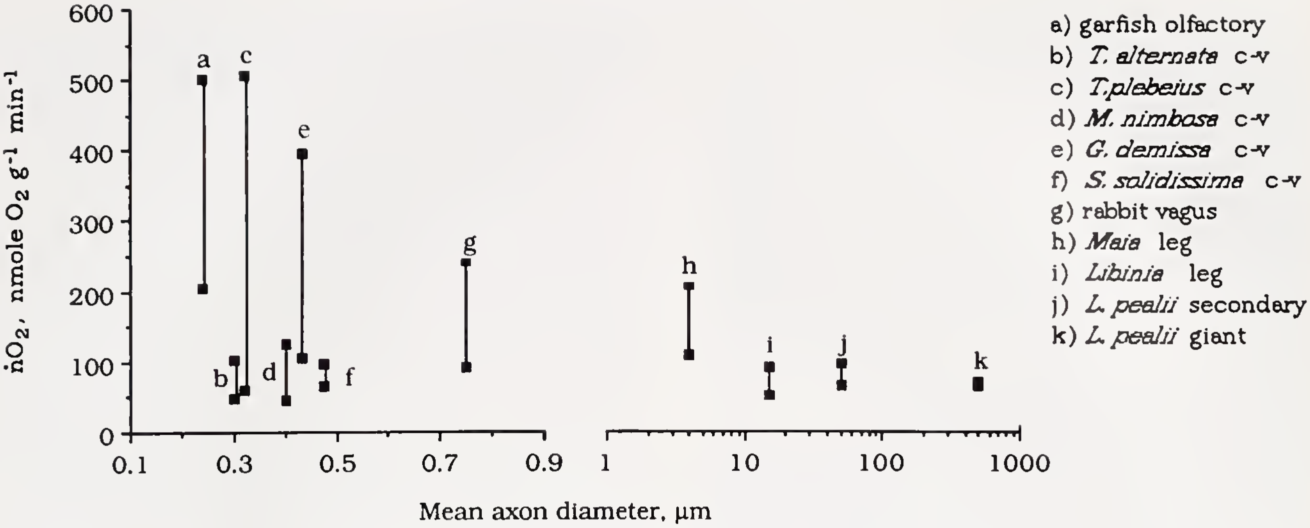

Figure 4. The effect of mean axon diameter on the resting and active oxygen consumption rates of nerves. Lower points refer to resting rates, upper points refer to active rates. Note the break in abscissa scale to accomodate a large range of axon diameters. Note also that lines c and e refer to bivalve cerebrovisceral connectives without neurohemoglobin, lines b, d, and f refer to connectives with neurohemoglobin. (a) rate, Ritchie and Straub (1979); diameter, Easton (1971); (b–f) rate, this study; diameter of b, c, e, f, Kraus *et al.* (1988) and d, estimated from other connectives; (g) rate, Ritchie (1967); diameter, Keynes and Ritchie (1965); (h) rate, Gerard (1932); diameter, estimated from Abbott *et al.* (1958); (i) rate, Baker and Connelly (1966); diameter, estimated from Baker (1965); (j) rate, Connelly (1952); diameter, estimated from Bullock (1965); (k) rate, Connelly and Cranefield (1953); diameter, Connelly (1952).

The maximum oxygen consumption rate of active nerves might be low if the energy requirements for action potential conduction were met by mechanisms that did not involve the consumption of oxygen from the surrounding medium. For example, if the neurohemoglobin-bound oxygen supply supported action potential conduction of connectives in the respirometer, then their measured oxygen consumption rates might have been artificially lowered. However, these neurohemoglobins begin to unload oxygen *in situ* at an ambient PO_2 of less than 10 mmHg, approximately the PO_2 where the rate of oxygen diffusion falls below the rate of oxygen consumption (Doeller and Kraus, 1988). Because the PO_2 of the bathing seawater in the respirometer rarely dropped below 100 mmHg during active respiration, the neurohemoglobin could not have been deoxygenated under these conditions. In experiments where neurohemoglobin fractional saturation was monitored during action potential conduction, the neurohemoglobin remained 100% oxygenated under air-equilibrated conditions, regardless of stimulus rate. In air-equilibrated seawater, resting and active oxygen consumption rates were not affected by carbon monoxide blockade of neurohemoglobin oxygen binding. We conclude that neurohemoglobin is not involved in supplying oxygen to support electrical activity at high ambient oxygen tensions.

Action potential conduction could also be less oxygen-dependent if the neurohemoglobin-containing connectives derived cellular energy from an anaerobic metabolic source. Both the sea anemone and the lugworm (which do not possess neurohemoglobin) can endure complete anoxia for days and still conduct action potentials or display complex sensory reflexes upon intermittent stimulation (Mangum, 1980; Surlykke, 1983). However, continuous action potential conduction by bivalve connectives with and without neurohemoglobin is quite sensitive to a depletion of both ambient and neurohemoglobin-bound oxygen. Under anoxic conditions, both the change in action potential amplitude of neurohemoglobin-containing connectives and neurohemoglobin deoxygenation follow the same time course (Kraus and Colacino, 1986; Doeller and Kraus, 1988). Connectives without neurohemoglobin and connectives with carbon monoxide neurohemoglobin both cease to conduct action potentials within 5–10 minutes after exposure to anoxic conditions (Fig. 3). Therefore, anaerobic metabolism cannot support action potential conduction at 1–2 pulses per second during anoxic conditions. Instead, the aerobic energy requirements of action potential conduction may be fundamentally different in the two types of connectives. Connectives with neurohemoglobin may consume less oxygen because they hydrolyze less ATP, assuming oxidative phosphorylation is equally efficient in both types of connectives.

Because the principle consumers of ATP in nervous tissue are the membrane-bound ionic pumps, less ATP would be hydrolyzed if fewer ions were pumped after each action potential. This would occur if fewer ions crossed the membrane during each action potential. There are several mechanisms which might lead to a reduction in the quantity of ions exchanged per impulse, each of which requires investigation. First, the total concentration of participating ions within the connective could be reduced. Cerebro-visceral connectives of the osmoconforming mussel *Mytilis edulis*, acclimated to 25% seawater, showed an approximately 50% reduction in internal sodium and potassium concentrations (Willmer, 1978a), but resting and action potential amplitudes were identical to those from animals acclimated to 100% seawater (Willmer, 1978b). Reduced ionic concentrations may be maintained in part by solute substitution to maintain osmotic balance, a diffusion barrier, or accessory ionic regulation by glial cells. Solute substitution may involve amino acids or other solutes, as occurs in the osmoconformer *Elysia chlorotica* (Pierce *et al.*, 1983; Parker and Pierce, 1985). The densely laminated perineural sheath of neurohemoglobin-containing connectives (Kraus *et al.*, 1988) may resist the diffusion of ions in a manner similar to the perineural sheath of cerebro-visceral connectives in two freshwater mussels, which restricts the passage of sodium ions (Twarog and Hidaka, 1972). Because a diffusion barrier alone is not sufficient to maintain low internal ion concentrations, supplementary ion pumping, perhaps at the peripheral glial cell layer, may be necessary. To support axon function during action potential conduction, glial cells may regulate the ionic constituents of the intercellular fluid by limiting the rise of potassium (Somjen, 1975; Orkand, 1982) or replenishing depleted sodium (Sattelle and Howes, 1975). Regulation of this type may be essential in closely packed axon bundles where intracellular volume is considerably greater than intercellular volume (see Fig. 3, Kraus *et al.*, 1988; intercellular volume of cerebro-visceral connectives of *Mytilis edilus* is only 12–20% of total volume, Willmer, 1978a). Ionic regulation by the neurohemoglobin-containing glial cells may be enhanced by the greater surface area contact between glial cells and axons (Kraus *et al.*, 1988). Glial cell function may be an important factor in the reduction in active oxygen consumption rates.

Second, fewer ions may be involved in action potential conduction if the axon membranes possessed a lower ion channel density. Willmer (1978c), measuring tetrodotoxin sensitivity, showed that connectives from *Mytilus edulis* may undergo a seven-fold reduction in sodium channel density upon acclimation to 25% salinity. Hypoxia-tolerant tissues may reduce the density of functional ion channels to compensate for less ion pumping during hypoxic or anoxic conditions (Hochachka, 1986). The increased membrane resistance resulting from a lower ion channel density might not effect the cable properties of the axons if the longitudinal resistances of the extracellular fluid and axoplasm were increased as a result of lower ion concentrations. Therefore, action potential properties would not be affected. As has been noted, the compound action potential properties of connectives with and without neurohemoglobin are not significantly different (Kraus *et al.*, 1988).

The lowered maximum oxygen consumption rates enable connectives with neurohemoglobin to effectively use the oxygen stored on the neurohemoglobin. The maximum oxygen consumption rates of connectives from both *T. alternata* and *S. solidissima* are well-matched to the linear oxygen unloading rates of each neurohemoglobin: compare 101 ± 40 to 146 ± 38 nmole O_2 g^{-1} min^{-1} for the oxygen consumption rate and neurohemoglobin unloading rate of *T. alternata*, and 96 ± 14 to 100 ± 24 nmole O_2 g^{-1} min^{-1} for the oxygen consumption rate and neurohemoglobin unloading rate of *S. solidissima* (Doeller and Kraus, 1988). Higher consumption rates would deplete the neurohemoglobin oxygen store more rapidly under anoxic conditions and lower rates would allow some oxygen to diffuse out of the connective.

The ecophysiological significance of a nervous system that can function under anoxic conditions for an extended period of time may become apparent when one considers the lifestyles of the bivalves studied here. [The following descriptions are from Stanley (1970) and personal observations in the field and laboratory.] *Tellina alternata* is a smooth shelled, laterally compressed bivalve with rapid burrowing capabilities. It typically resides 10–20 cm below the sediment surface in sandy mud near the mean low tide mark of low energy beaches where much of the organic debris carried by swash currents tends to settle out. The sandy mud of its habitat is black to grey and smells strongly of sulfide, which is characteristic of anoxic sediment. Other species commonly found in this habitat are *Chaetopterus variopedatus*, *Notomastus lobatus*, and *Divariacella quadrisulcata*.

As a deposit feeder, *T. alternata* siphons organic particles from the sediment surface. During feeding, the animal has a ready supply of oxygenated water as it extends its long incurrent siphon to the sediment surface. However, once the sediment around its siphon is depleted of deposits and the animal retracts its siphon to burrow laterally to a new location, its source of oxygen is removed. Burrowing requires much foot and valve movement coordinated by an operational nervous system. Under anoxic conditions, *T. alternata* maintains a high metabolic rate (Kraus, 1986), most likely by using the anaerobic metabolic pathways found in many bivalve molluscs (de Zwaan, 1983). Therefore, an aerobic nervous system which is able to operate for 20 minutes or more under anoxic conditions by using a neurohemoglobin oxygen store would allow *T. alternata* to burrow laterally for a distance of at least 1 m, at the observed rate of 5 cm/min, certainly far enough to locate fresh surface deposits.

Spisula solidissima is a large laterally compressed active clam. Although *S. solidissima* is a filter feeder with relatively short siphons, it does not inhabit a permanent burrow and often burrows rapidly through the sediment below the surface. Under these hypoxic conditions, its neurohemoglobin-containing nervous system could continue to operate to ensure the eventual return to a source of oxygen. *Macrocallista nimbosa* exhibits a morphology and lifestyle similar to *S. solidissima* and its neurohemoglobin probably operates in a similar fashion. Neither *S. solidissima* nor *M. nimbosa* burrow as deeply as *T. alternata* and their neurohemoglobins are not as concentrated.

Tagelus plebeius and *Geukensia demissa* are less active bivalves than *T. alternata* and *S. solidissima*. *T. plebeius* is an elongated cylindrical filter-feeding bivalve that burrows slowly. It is commonly found in sandy intertidal mud flats in communities with *Mercenaria mercenaria* and *Saccoglossus kowalewskii*. *T. plebeius* occupies a permanent cylindrical burrow which extends vertically to 50 cm or more below the sediment surface. At high tide, *T. plebeius* occupies the upper portion of the burrow and extends its siphons through two small openings to filter feed from the abundant food source in the overlying water column. As the tide ebbs, the animal withdraws its siphons to avoid predation and slowly moves down in the burrow to follow the water table. Under anoxic conditions, *T. plebeius* exhibits very low rates of heat production and oxygen consumption until it is reexposed to aerated conditions (Kraus, 1986). *Geukensia demissa*, another sedentary bivalve, is found securely attached by byssal threads to stationary debris near the sediment surface in backwater regions. During air-exposure, animals extract oxygen from the air, but deep tissues remain poorly oxygenated (Booth and Mangum, 1978).

In summary, the cerebro-visceral connectives of *T. alternata* and *S. solidissima* with neurohemoglobin, and *T. plebeius* and *G. demissa* without neurohemoglobin exhibit electrophysiological and ultrastructural characteristics which are similar to each other and to other nerves with small diameter axons (Kraus *et al.*, 1988). However, the connectives with neurohemoglobin consume much less oxygen during action potential conduction than connectives and other nerves without neurohemoglobin. This increased efficiency enables the neurohemoglobin-containing connectives to effectively use the neurohemoglobin oxygen store, which in turn may enable the animals to use continued neuromuscular activity during hypoxic and anoxic conditions.

Acknowledgments

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A Model of a Temporal Filter in Chemoreception to Extract Directional Information From a Turbulent Odor Plume

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Abstract. We ask whether animals can derive spatial information from temporal patterns contained in turbulent odor plumes under realistic biological constraints of receptor properties (size and physiological responses) and behavioral requirements (time averaging). We modeled an appropriately scaled aquatic odor plume with a salt tracer to serve as the input to two different lobster chemoreceptor organs.

We then constructed a computer model based on some of the currently known temporal filtering characteristics of lobster chemoreceptor cells *in situ.* The output of this model represents the supra-threshold stimulus intensity fluctuations "seen" by realistically adapting cells. The input and output of the model were evaluated for directional information. We focused on four parameters that characterize concentration peaks within the plume: *height, length, maximum rising slope,* and *off time* (time between peaks). These characteristics were analyzed under two biologically important sampling strategies: one corresponding to a *continuous*-sampling receptor organ (*e.g.,* lobster leg, catfish nose) and the other to a *discrete*-sampling receptor organ (*e.g.,* lobster nose, tuna nose). We let the discrete-sampling model analyze at a frequency of four sniffs per second, each averaging over 100 ms. The continuous-sampling model used an historic exponential average of 25, 100, or 1000 ms based on disadaptation rates of receptor cells *in situ.*

In this preliminary study, filtered odor spectra contained less biologically useful information than the unfiltered input spectra. Discrete and continuous models were not different. In all cases, the probability distribution of maximum rising slopes of stimulus concentration contained the most reliable directional information.

Introduction

Various terrestrial and aquatic animals show remarkable abilities to orient in turbulent odor plumes. Considering the non-directionality of odor signals *per se,* we hypothesized that turbulent odor dispersal processes might create spatial patterns that could serve as directional cues, and that chemoreceptor organs may have temporal filter properties that match dominant spatial frequencies of odor plumes at size scales relevant to the animals and their receptor organs (Atema, 1985, 1988; Derby and Atema, 1988). Dispersal of an odor into a fluid volume occurs over a wide range of size scales. At size scales less than 1–10 mm, molecular diffusion determines the distribution of odor in the environment, whereas at scales larger than 1–10 mm advection dominates the dispersal process. Aquatic systems are likely to be turbulent at scales larger than 10 mm. This is the size scale of most macroscopic animals.

While turbulence is chaotic and thus not predictable at any instant in time or space, it has predictable patterns when spatial and/or temporal averages are taken. Mean distributions of turbulent odor dispersal can be predicted by Sutton's model (1953). This model is based on a continuously and constantly emitting odor source and assumes that the average odor concentration will follow a normal (Gaussian) distribution in any plane perpendicular to the down-current axis. The time-averaged odor plume increases in area and decreases in concentration exponentially with distance from the source. This model describes a continuous (non-patchy) odor distribution

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within the plume. To establish a reasonable approximation to a Gaussian distribution under the conditions considered by Sutton, an averaging time of several minutes is required (Pasquill, 1961; Gifford, 1968; Miksad and Kittredge, 1979; and Elkinton, *et al.*, 1984). Predicting animal responses from such time-averaged models has proven inadequate in some cases; when orientating toward the source of a pheromone plume, gypsy moths did not appear to time-average on the same scale as the Sutton model (David *et al.*, 1982; Elkinton *et al.*, 1984). It is obvious that animals do not always have the "luxury" of waiting a few minutes to take an average! They may have to make behavioral decisions in as short a time period as the circumstances demand. This requires analysis of more instantaneous parameters of odor plumes.

The instantaneous distribution of odor within a turbulent odor plume is quite different from Sutton's (1953) time-averaged distribution (Wright, 1958; Aylor, 1976; Aylor *et al.*, 1976; Shorey, 1976; and Murlis and Jones, 1981). Instantaneous plumes meander, and break up into filaments and patches. Instantaneous odor distributions follow local environmental turbulence. Turbulent eddies occur simultaneously over a range of sizes. The largest eddies pass down their energy into smaller and smaller eddies until the energy finally dissipates. Eddies of the size scale of an odor filament cause it to break up into patches; smaller eddies redistribute the odor within the patches and cause decreases in the odor concentration gradients at patch edges; larger eddies move the entire odor plume and cause meandering. For a more complete description of Gaussian and instantaneous odor plumes see Elkinton and Cardé (1984). The result of a patchy odor distribution is that an animal located downwind of an odor source will experience periods of odor concentration well above and below the mean concentration.

Measurements of the instantaneous structure of odor plumes are needed to understand which dynamic characteristics of the plume are the best indicators for orientation within the plume. These measurements must be made at spatial and temporal sampling scales an order of magnitude greater than those relevant to chemoreceptors or the animal behavior under consideration: different animals and different receptor organs have their own characteristic time and space scales (Atema, 1985, 1988). At present, spatial scales for receptor organs must be estimated from their morphology and in some cases from preliminary data on flow fields (Vogel, 1983; Moore and Atema, unpub.). Temporal scales relevant to chemoreceptor organs are poorly known; they must be estimated from still-rare physiological response data (Kaissling *et al.*, 1987; Voigt and Atema, 1987a, b; Christensen and Hildebrand, 1988). Eventually, models must be adjusted to accommodate these species-specific scales.

Our study was designed to measure odor dispersal at the sampling scales of two lobster (*Homarus americanus*) chemoreceptor organs, the antennules (olfaction) and legs (taste). To model an odor plume at a size scale of relevance to a lobster we chose a velocity and volume similar to the filter feeding current of a single mussel *Mytilus edulis*, a common prey of lobsters. A filter-feeding mussel generates a turbulent odor plume of metabolites; this plume is carried away and redistributed by ocean currents. The pumping rates of *M. edulis* range from 0 to 50 ml/min (Kirby-Smith, 1972; Winter, 1978). The model plume was generated by continuous injection of a salt tracer ("the mussel") in a fresh water flume ("the ocean").

For *H. americanus*, odor cues appear to be more important than rheotaxis in orientation to distant odor sources (McLeese, 1973). Since removal of one lateral antennule results in random direction choice, directional decisions seem to be based on a comparison of input from the two lateral antennules (Devine and Atema, 1982). Similar results were obtained in odor orientation experiments with the spiny lobster, *Panulirus argus* (Reeder and Ache, 1980).

Flicking of the lateral antennules decreases the boundary layer around the dense clusters of 1 mm long aesthetasc sensilla (Snow, 1973; Moore and Atema, unpub. obs.) and appears to be the functional equivalent of vertebrate sniffing. Flicking probably determines the spatial and temporal frequency filtering of the chemoreceptor cells of the antennule (Schmitt and Ache, 1979; Atema, 1985). *H. americanus* antennules can flick with burst rates of 4 s^{-1} (pers. obs.). Once in the general vicinity, lobsters use their legs to locate and recognize food; for this, leg chemoreception is essential (Derby and Atema, 1982). Legs wave but do not flick: they seem to sample odor plumes continuously.

The exact sampling frequency and volume relevant to lobster chemoreceptors are not yet known. Temporal filters result from boundary layer plus cellular adaptation (Borroni and Atema, 1987) and disadaptation rates (Voigt and Atema, 1987a, b). From these data and response rates obtained in insect pheromone receptor cells (Kaissling *et al.*, 1987; Christensen and Hildebrand, 1988), we estimate that lobster leg receptors might follow pulse rates of 0.1–10 s^{-1}. Spatial scales are estimated to be on the order of 1–10 mm. To resolve slopes of concentration peaks, *i.e.*, rate of increase of odor concentration, we sampled the odor plume at 40 s^{-1}, an order of magnitude faster than the estimated sampling (*i.e.*, flicking) frequency of our biological filters under study.

We analyzed our "model plume" with the two different sampling methods: *continuous i.e.*, the slow movements of a lobster leg, and *discrete i.e.*, the fast flicking of lobster antennules. These two sampling methods also

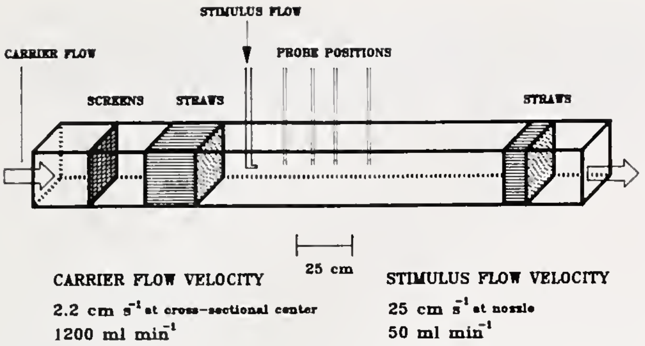

Figure 1. Diagram of flume used for salt plume turbulence measurements at the size and time scale of lobster chemoreceptor organs. Probes shown at each of the four sites; only one probe was in the water during the measurements, all taken in the cross-sectional center of the plume.

exist in the functional designs of fish noses. Fish have been classified (Døving *et al.*, 1977) as isosmates (continuous ciliary flow, *e.g.*, catfish) and cyclosmates ("sniffing" via accessory pumps and muscles, *e.g.*, tuna). Overall, our model was designed to represent the stimulus pulse patterns that a chemoreceptor cell *in situ* with known temporal filter characteristics might detect within the odor plume emanating from a pumping mussel located a short distance upcurrent. The first generation model is meant to define critical parameters, particularly those for which little or no information is yet available.

Materials and Methods

Plume model

To generate the model plume we used a 248 cm × 34 cm × 23 cm flume with a tap water flow (1.2 ± .21/min) (Fig. 1). This carrier flow passed through three sheets of window screen, and a collimator of 5 mm diameter soda straws 22 cm long. The test area was 142 cm long. Another row of 10-cm long soda straws was placed before the outflow tube. The flow velocity in the center of the test area was 2.2 ± 0.2 cm/s measured by timing the movement of a patch of dye.

The tracer plume was a 0.7% NaCl solution injected at 50 ± 1 ml/min. into the test area of flow through a Pasteur pipette with 1 mm ID tip opening. Flow velocity at the nozzle was 25 cm/s (Re ≈ 500). The nozzle of the pipette was 10 cm down-stream from the collimator, 7 cm from the bottom and equidistant from the sides. A dye was mixed in with the salt solution to visually locate the plume center line. Although the tracer was somewhat denser than the surrounding water, it did not drop significantly over the sampling area.

As an indicator of instantaneous salt concentration we measured conductivity (YSI model #35) around two silver wire (gauge 30) electrodes each with an exposed length of 1 cm. The wires were spaced 1 cm apart to give a spatial sampling area of 1 cm^2. These dimensions were chosen to approximate the 1 ml estimated sample volume of one lobster flick, or the slow flow volume immediately surrounding a lobster leg. The output from the conductivity meter was monitored on a chart recorder (Gould Brush model #220) and recorded on an FM tape recorder (Vetter model D) for later computer analysis.

Salt concentrations were measured at four sites, each in the center line of the plume: 12.5, 25, 34.5, and 50 cm from the pipette mouth. The plume center line was located by first sighting the dye. Once the probe was positioned it was left there for 1.5 min before recording a 7.5 min sample. One 7.5 min sample was taken at each site.

The four 7.5-min analog samples were replayed from the tape recorder through an AD converter into a IBM PC computer. The sampling rate of the computer was set at 40 s^{-1}, *i.e.*, the computer sampled the tape every 25 ms for 9 ns to derive a data point. Although the 40 s^{-1} rate was chosen to be 10× the lobster flicking sample rate it also represented, perhaps fortuitously, the maximum frequency of turbulence in the plume without including high frequency noise of the recording system.

A set of known and fixed salt concentrations were recorded and played through the AD converter to construct a calibration curve for the entire salt concentration recording system. This allowed the computer voltage data to be expressed as actual salt "stimulus" concentrations.

The salt plume appeared as a series of concentration peaks of varying strength and duration. They represent a typical turbulence spectrum (Murlis and Jones, 1981; Atema, 1985). As expected the peaks flattened with distance downstream from the source (Fig. 2). These turbulence spectra were used as the input data for our receptor filter model.

Receptor filter model

For the discrete-sampling "nose" we assumed that the receptors will average the sample over a period of 100 ms (approximate time for the down-stroke of the antennule) and then "wait" 150 ms while the antennule recovers for the next downstroke. Preliminary observations (unpub.) showed that the down-stroke thoroughly mixes the odor distribution in the sampling space. Therefore, we used a centered arithmetic mean of the concentrations encountered over the 100 ms sample period. For the continuous sampling nose, we assumed it averages over 25 ms.

Receptor cells adapt and disadapt as they sample. For initial simplicity, we base this model on data from self-adaptation experiments, *i.e.*, adaptation to the same compound(s). The instantaneous adaptation state of a

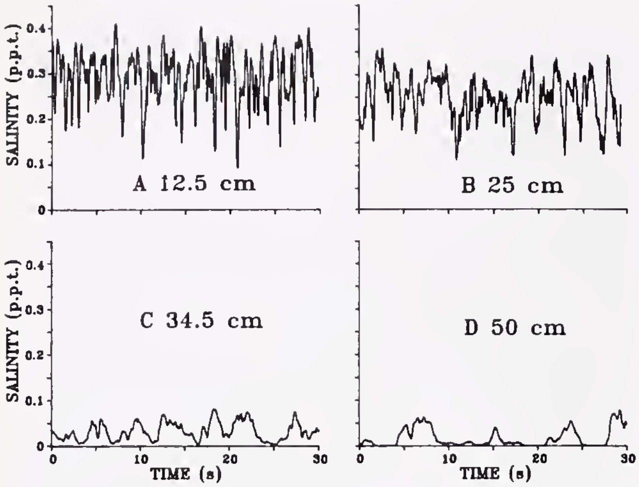

Figure 2. Typical turbulence spectra of salt concentrations in the plume. Random 30 s segments from 7.5 minute recordings, one at each site. A–D: Distances from the pipette mouth to the plane of the electrodes. The plume changes its turbulent character rather abruptly between 25 and 34.5 cm. It is not until 50 cm from the source that the larger scale turbulence causes sufficient meander to result in periods of zero signal.

receptor cell is measured as its response threshold and is dependent on past concentrations sampled. We assumed that receptor cells self-adapt within seconds (see Borroni and Atema, 1987, and in prep.) and for this model we use instantaneous and complete adaptation immediately following any sample period (*i.e.*, 100 ms for discrete sampling and 25 ms for continuous sampling). Previously encountered concentrations will have cumulative effects on the current adaptation state of the cell. We considered a total period of effectiveness of T = 10 s, an estimate based on the disadaptation time course of taurine and glutamate receptor cells *in situ* which range from 2.5–40 s for complete disadaptation from a 1-s glutamate or taurine pulse 1–3 log steps above a taurine or glutamate background (Voigt and Atema, 1987a, b). The magnitude of these effects depends on the magnitude of the previously sampled concentrations and the time (t in s) since their occurrence. We assumed that the effect of a previous concentration upon the cell's instantaneous adaptation state decays exponentially with time. Based on the presumed disadaptation time course of taurine and glutamate receptor cells *in situ,* we generated equation 1 to determine the weight factor (W_t) by which the response of the cell at t = 0 should be reduced due to each of the 40 samples in the previous period (T = 10 s).

$$W_t = \exp(1 + 0.4 \times (T - t))/100 \qquad (1)$$

The numbers 1, .4, and 100 were chosen to scale the height and rate of decay of the curve to the height and time scale of the input data. This model assumes that all previous samples contribute to the current adaptation state of the receptor cell—even those that did not result in output from the cell (*e.g.*, Fig. 3, subthreshold sample bar at t = −2, etc.). In addition, for the sake of simplicity, we used the unmodified input data as the basis for each new point. (In doing so we did not take into account possible interactive effects that may exist between samples, *e.g.*, strong pulses early in the period T reduce the stimulating effectiveness of pulses later in the period T due to biochemical adaptation of the cell; if adaptation is a result of excitation, these strong pulses may then reduce the effectiveness of later pulses in suppressing the response at t = 0).

W_t was used as the weight factor in a "historical" weighted mean (A) in equation 2:

$$A = \Sigma(C_t \times W_t)/\Sigma W_t \qquad (2)$$

where A is the adaptation state of the receptor at time t, and C_t is the stimulus concentration at time t. The weight portion ($W_t/\Sigma W_t$) of equation 2 is visualized in Figure 3 (dotted curve). This curve reflects the amount of adaptation (= threshold increase) that the 40 sample bins prior

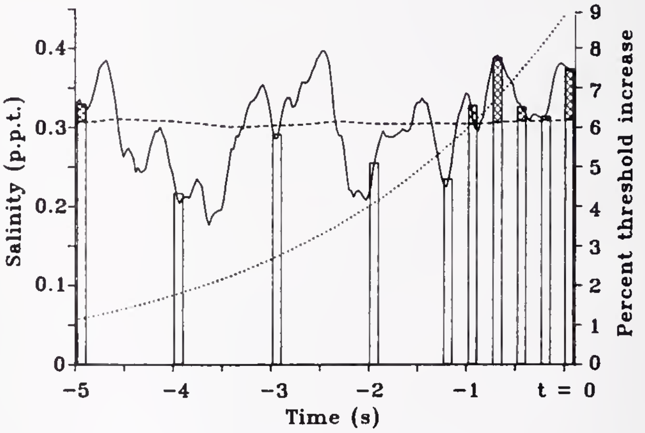

Figure 3. Five second sample of salt plume concentration spectrum (solid curve; salinity in ppt) and sampling bins (vertical bars) from discrete sampling model. Total height of a bar represents mean unfiltered concentration encountered during sample period; width of bar represents sampling time bin (one flick = 100 ms). Cross-hatched portion of bar represents perceived concentration, *i.e.*, above the cell's instantaneous threshold (= adaptation level: broken line) caused by previous samples taken. The model assumes constant flicking at 4 s^{-1}; for clarity not all sample bins are shown, except in the last 1.5 s. Only sampled concentrations contribute to adaptation; concentrations in between sample periods go "unnoticed." In this example, a hypothetical cell with 10 s disadaptation time is used; the exponential function (dotted line) represents the time-dependent amount of suppression (% of response at t = 0) caused by preceding stimuli: recent samples (*e.g.*, t = −1) have greater effects (6.2% of sample at t = −1) on the response at t = 0 than earlier samples (*e.g.*, t = −5; 1.1% of sample value at t = −5); samples prior to t = −10 no longer affect the response to a sample at t = 0.

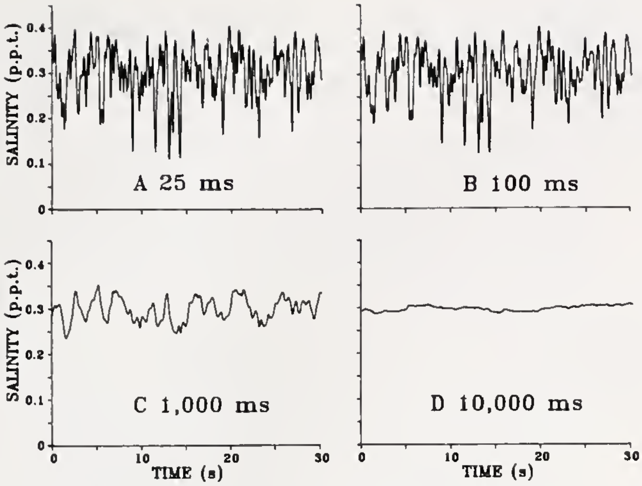

Figure 4. Concentration spectra from the 12.5 cm sample site (Fig. 2A) filtered with different disadaptation times. A. Unfiltered spectra (from Fig. 2A). B, C, D. Spectra representing instantaneous adaptation state of these different receptor cells with very fast (B), fast (C), and slow (D) disadaptation characteristics. Disadaptation time is based on the assumptions of instantaneous adaptation and an exponential disadaptation time course as shown in Figure 3. The 10,000 ms filter results in a smooth concentration average similar to the broken line of Figure 3.

to t = 0 contribute to the overall adaptation state at t = 0. In Figure 3 we visualize a series of "flicks" as they sample the input concentration profile (solid line). The response to a pulse at t = 0 is affected by the cell's exposure to a series of previous concentrations (measured in 100 ms sample bins every 250 ms for 10 s). The effect of each of the previously sampled concentrations is given by an exponentially decaying function from t = 0 to t = −10 s. For example, the sample bar at t = −5 registered 0.33 ppt salinity; it raises the threshold at the t = 0 sample by about 1% or 0.0033 ppt. The sample at t = −1 registered about the same salinity but, being more recent, its effect on raising the threshold at t = 0 is 6% or 0.0198 ppt. The sum effect of all samples during T causes the cell's threshold to be raised: the hatched portion of the bar is supra-threshold. The threshold curve (broken line of Fig. 3) represents a moving average of points generated as described above.

For the continuous sampling filter model we assumed that the receptor cell integrates over a fixed (*e.g.*, 25 ms) time bin, then integrates over the immediately following 25 ms bin, etc. The samples taken in previous time bins influence the response to the sample at t = 0 as in the discrete sampling model except that now the bins follow each other directly. We chose disadaptation times of T = .1, 1, and 10 s for the continuous sampling model. Thus, with the input data sampled every 25 ms, the .1 s (100 ms) disadaptation time bin contains four points, 1 s contains 40 points, and the 10 s contains 400 points (Fig. 4).

To analyze peak characteristics of the odor plume we had to define a peak and assign a baseline, *i.e.*, a stimulus background level to which a real receptor cell would be adapted. The background is evident when stimulus concentrations return to undetectable levels after a burst of stimulus pulses (Fig. 2D). However, when pulses continue to follow so rapidly that stimulus concentrations remain elevated between pulses (Fig. 2A, B) we must adopt a quantitative rule for a background. We chose the background levels that result from integration over 10 s, *i.e.*, the cell was assumed to see peaks superimposed on one of the concentration averages of Figure 5. A peak is then defined as starting and ending at the appropriate background. In addition, only when the concentration value between two peaks dropped below 30% of the height of the previous peak, were the peaks considered separate.

For both the discrete and continuous model, four stimulus peak parameters were measured: *height*, from the maximum value of the peak to background; *length*, from beginning to ending of peak as defined above; *off time*, time from the end of one peak to the beginning of the next peak; and *maximum slope* on the rising side of the peak, calculated as the maximum value of the double linear concentration-time tangent, measured as ppt per 25 ms sample bin. Examples of these are shown in Figure 6.

Results

Since the number of samples taken for making a directional decision by animals orienting in a plume might

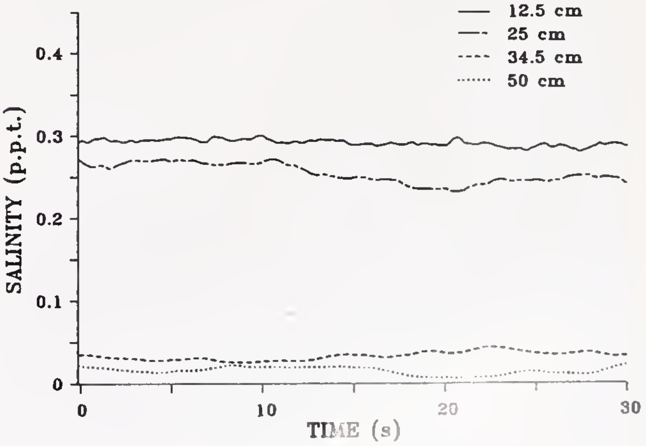

Figure 5. Concentration spectra at each of the four sample sites (Fig. 2) at an averaging time of 10,000 ms (10 s). This low-pass filter results in a "mean stimulus concentration." These means represent the cell's response threshold in the stimulus conditions of the four sites; they were used as baselines for peak measurements.

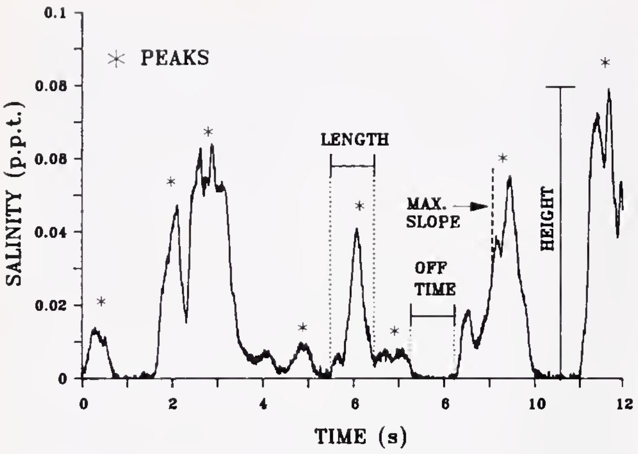

Figure 6. Unfiltered salt plume spectra from 50 cm site demonstrating the peak parameters analyzed. *Peak height*, measured against a baseline (Fig. 5) reflecting the presumed adaptation state of the receptor (baseline shown here: zero). *Peak length*, time from the beginning to ending of peak (see text). *Off time*, time of no signal, between successive peaks. *Peak slope*, maximum slope on the rising side of the peak.

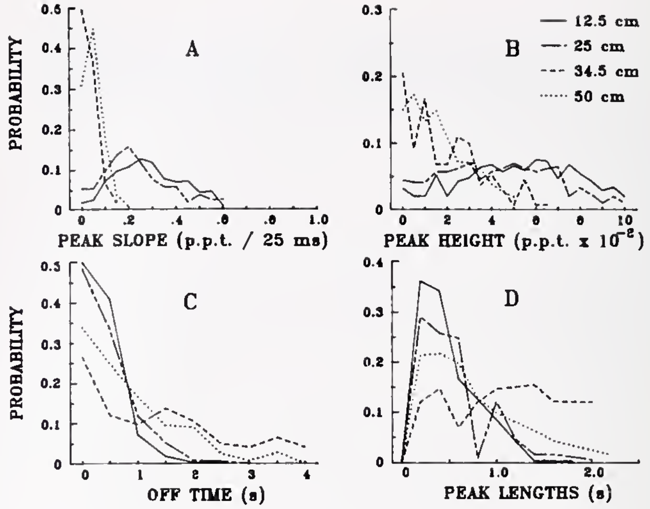

Figure 7. Probability distributions of peak parameters from the discrete sampling model with a very fast disadaptation time of 100 ms. A. Peak slope B. Peak height C. Off time D. Peak length.

be small (for chemoreception in the order of seconds) compared with our 7.5 min sample, we plotted probability distributions of the four peak parameters. The distributions were calculated over the entire 7.5 min sample. Probability distributions reveal those characteristics of an odor plume that change most reliably with distance, and hence might provide directional information. The probability distributions of peak parameters from the discrete sampling model (Fig. 7) show that the most reliable source of directional information is contained in the maximum slope distribution (Fig. 7A). Approximately 85% of the slope values encountered at the closest two sites are not encountered at the farthest two sites. The closest two sites have more sharply rising slopes, while the other two sites have shallower slopes. The two near sites have nearly indistinguishable characteristics as do the two far sites. We know nothing about the slope discrimination ability of the actual chemoreceptor system.

The peak height distribution (Fig. 7B) provides less directional information than peak slope distribution. Peak height values at the farthest two sites range from 0–0.06 ppt. This same range of heights occurs 65% of the time at the closest two sites. This means that directional information is present in only 35% of the peak heights encountered; and this does not take into account the inaccuracy of height discrimination present in the receptor system. This overlap in the probability distributions is also present in the off times, 70%, (Fig. 7C) and peak lengths, 60%, (Fig. 7D).

Similar results were obtained in the continuous flow model (Fig. 8). The greatest separation in distributions among the four parameters occurs with the peak slopes (Fig. 8A). The overlap among the four sites is approximately 15%. The overlap in the distributions of the other three parameters is 73% (peak heights), 70% (off time), and 48% (peak lengths) (Figs. 8B, C, and D, respectively).

The probability distributions of peak slopes for different disadaptation times (25, 100, 1000 ms) reflect, as expected, that concentration peaks become smoother as the disadaptation time of the receptor cell *in situ* increases (Figs. 9A, B, C). However, this overall change in slope does not change the relative amount of overlap of the four distributions; this stays surprising constant around 15%. Since this overlap stays constant with

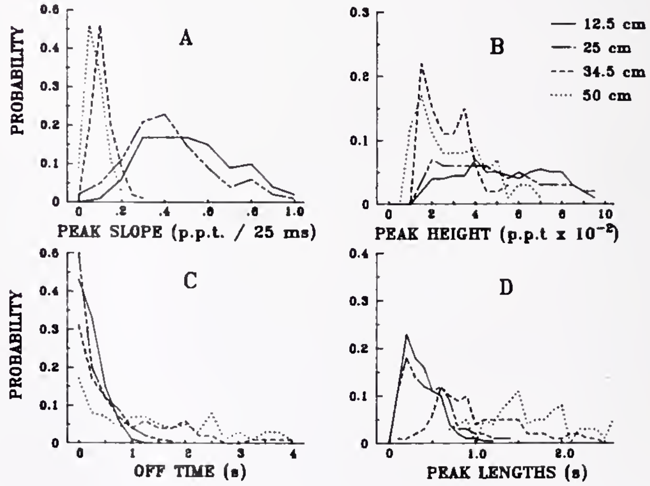

Figure 8. Probability distributions of the four peak parameters from the continuous sampling model with a disadaptation time of 100 ms (Fig. 5B). A. Peak slope B. Peak height C. Off time D. Peak length.

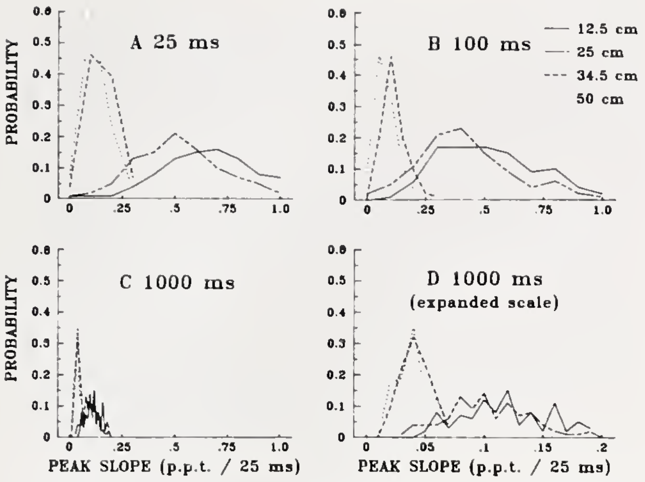

Figure 9. Effect of averaging time on the probability distribution of maximum peak slopes representing chemoreceptors with different disadaptation times (A, B, C) from the continuous flow model. D is an expanded version of C.

changing disadaptation times, the potential directional information content of the parameter stays constant also. The same was found for the other three peak parameters. Therefore, receptor cells with different averaging periods cannot improve on this situation.

Discussion

This study accomplished two goals. First, we measured the turbulent spectrum in an odor plume scaled such that it might be realistic for a lobster searching for an odor source. Both the spatio-temporal scale of the plume itself and the spatial average taken by the "receptor," *i.e.*, the electrode size and spacing, were lobster-like. Second, we constructed a first-generation temporal filter model that reflects the biological reality that receptor cells adapt to ambient concentrations where electronics do not unless specifically instructed. Our instructions were based on preliminary physiological data on self-adaptation and disadaptation rates of chemoreceptor cells *in situ*, *i.e.*, including the boundary layer normally present. The filter model must be adjusted as physiological data and boundary layer measurements accumulate.

The turbulent spectrum of any plume is highly dependent upon the physical conditions of the environment. To develop this first model, we chose one specific plume condition: a constantly pumping mussel under one laminar carrier flow condition and we sampled only at four locations. In the future, extensive sampling must be done under various flow conditions and with different odor sources encountered in the natural environment of the *H. americanus*.

As the plume ages the physics of turbulent dispersal changes the values of plume parameters, *i.e.*, peak slopes get less steep, peak heights fall, *etc.* From a spatial comparison of these changes, animals could estimate source distance and direction. Distance information might be used to assess if it is energetically worthwhile (considering hunger, predators, *etc.*) to proceed to the source or to stay put: the longer a plume has been in the environment the higher the probability that the source is gone, *e.g.*, a competitor may have found and exploited the source. In addition, animals that have no earth reference (such as visual or contact cues of the ground) to determine current direction may use spatial sampling of turbulent plumes to determine source direction. Our filter model can be used to analyze which properties of turbulent plumes and which physiological and behavioral sampling strategies provide the best information.

The strength of any model lies in its ability to rigidly define one's assumptions and hence show which assumptions do not correspond with biological or physical reality. One of these inconsistencies may already be apparent. We suggested (Atema, 1985, 1987, 1988) that temporal filter properties of chemoreceptor cells may be matched to those dominant spatial frequencies of turbulence that contain important biological information. We had thus expected that filtered turbulence spectra would contain more directional information. Instead we found less separation in the filtered probability distributions of peak slopes than in the unfiltered distribution (Fig. 10). Since the biological assumption of matching of filters seems reasonable and is found in many other sensory systems we must first examine and refine the model before concluding that chemoreceptors are exceptionally constrained by the physics of the microenvironment (De Simone, 1981) or perhaps by the biochemical processes of sensory transduction.

Various assumptions must be re-evaluated in light of

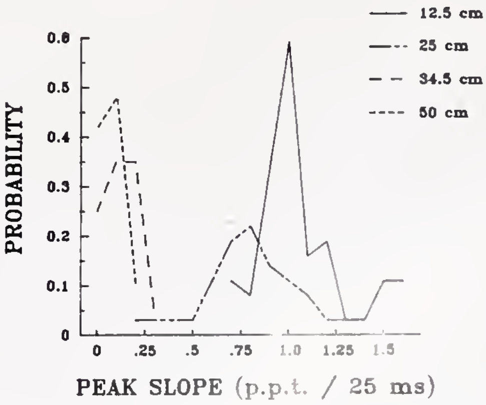

Figure 10. Probability distribution of unfiltered peak slopes (Fig. 2) continuously sampled every 25 ms at the four sample sites, against an absolute 0.02 ppt salinity threshold.

future physiological results. There is insufficient knowledge of the adaptation and disadaptation time courses of various chemoreceptors and, in particular, on the contribution of the boundary layer. This affects the adaptation level of the cells against which peaks are measured. "Peak height against background" may be a more important parameter for orientation than we conclude from the current model. Furthermore, if cell excitation determines its level of adaptation then we must revise the exponential function (1) we used here, and use response output, not stimulus input, as the basis from which to calculate adaptation state. Finally, we know nothing about the time over which chemoreceptor cells integrate. Some photoreceptors integrate over 500 ms under very low photon density conditions (Fein and Szuts, 1982). Our 25–1000 ms assumption for chemoreceptors may or may not be realistic. It corresponds to fast reaction times measured elsewhere (Kelling and Halpern, 1983). If boundary layers form the rate limiting factor determining response latency then data from very different chemoreceptor organs can be compared.

Chemoreceptor cells may function under rather severe physical constraints. Every interface between a solid and a fluid, be it air or water, results in a boundary layer (Tritton, 1977; Vogel, 1981). Close to the solid surface fluid motion approaches zero velocity. Chemical stimuli can penetrate through this inner portion of the boundary layer only by molecular diffusion. The thickness of this diffusion layer may be a critical constraint not only on response latency (De Simone, 1981) but also on the temporal resolution of chemoreceptor cells since diffusion time increases geometrically with distance. Latency and temporal resolution are obviously related. A diffusion layer will act as an integrating filter, and hence a low pass filter, reducing the frequency response of the receptor system to lower frequencies than those present in the free flow around the organ (Fig. 2). The critical difference between flicking and non-flicking may be reduction of the boundary layer by high-velocity flicks. The present analysis showed no significant difference in discrete and continuous sampling. This may change with more extensive analysis of different plumes and more refined assumptions. Measurements of boundary layers under biologically realistic conditions must be made, and the results added to this model as integrating filters with diffusion layer thickness as a parameter. To this must be added the subsequent diffusion distances from the surface of the receptor organ to the receptor site requiring accurate morphological measurements of receptor sensilla. The remaining filtering is then probably due to biochemical processes.

This model and its subsequent refinements can be used to describe and analyze different odor plumes extensively. This will give insight into those turbulent features that are most suitable for biological use given the intrinsic constraints of chemoreceptor physiology and morphology. Animal behavior may become involved as an animal searches an odor plume for useful features. In this context it may be interesting to refer to the sudden difference in turbulent spectra between the two very similar near sites and the two quite similar far sites (Figs. 2, 5, 7, 8, 9, 10). Such sudden breaks are not uncommon in turbulent plumes and may serve as useful biological indicators of nearness to the source. In general, we expect that this model will help us see to what degree the physics of the environment constrain the ability of chemoreceptors to extract spatial and temporal features of natural odor distributions that might be useful for orientation.

Acknowledgments

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Response Properties of Chemoreceptors from the Medial Antennular Filament of the Lobster *Homarus americanus*

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Abstract. We determined the spectral tuning properties of 53 single cells from the medial antennular filament of the lobster *Homarus americanus.* Test stimuli were 15 single compounds and a mixture that included all 15 test compounds. Three main cell populations were found: hydroxyproline best (14 cells), taurine best (13 cells), and arginine best (11 cells). Most hydroxyproline and taurine best cells were narrowly tuned and had no consistent next best stimulus. In contrast, arginine best cells were generally broadly tuned and had consistent second (leucine) and third (lysine) best stimuli. Mixture suppression occurred in most cells. Responses of hydroxyproline, taurine, and arginine best cells to the mixture were 25%, 38%, and 50%, respectively, relative to the responses of these cells to their best compound alone (100%). In a second experiment, we tested 12 arginine sensitive cells with a series of arginine analogs. Most cells were broadly tuned and as a population showed a similar response ratio to arginine, leucine, and lysine.

Introduction

Amino acid receptors occur in the olfactory and taste systems of numerous species. Crustaceans have proved to be particularly useful models for investigating properties of such external amino acid receptors. Behavioral studies have identified stimulatory amino acids and described responses to these compounds in marine, freshwater, and semi-terrestrial species (Carter and Steele, 1982; Zimmer-Faust *et al.,* 1984; Johnson and Atema, 1986; Tierney and Atema, 1988; review: Ache, 1982). These studies have generally supported the idea that amino acid chemoreception is related to the detection of food. Electrophysiological studies, particularly with lobsters and crayfish, have provided additional information on the location, sensitivity, and specificity of single amino acid receptors. Two significant properties of lobster chemoreceptors have been identified. First, unlike most chemoreceptors that function in feeding behavior in vertebrates and insects, lobster cells are typically narrowly tuned. Many receptors respond strongly to only one or a few compounds (Derby and Atema, 1982b; Johnson and Atema, 1983; Johnson *et al.,* 1984). Second, most crustacean chemoreceptors display mixture interactions in which responses to a single compound are reduced or enhanced when this compound is presented in a mixture (Johnson and Atema, 1983; Johnson *et al.,* 1985; Gleeson and Ache, 1985). The discovery of these properties has generated new ideas about how chemoreceptor systems may resolve stimulus quality (Atema *et al.,* 1988; Derby and Atema, 1988).

Electrophysiological studies of crustacean chemoreception have primarily focused on cells in the lateral antennular filament (Shepheard, 1974; Johnson and Ache, 1978; Johnson and Atema, 1983; Derby and Ache, 1984a; Gleeson and Ache, 1985; Johnson *et al.,* 1985; Spencer, 1986) and in the walking legs (Bauer *et al.,* 1981; Derby and Atema, 1982a, b; Johnson *et al.,* 1984; Hatt, 1984). However, other structures, including the medial antennular filament, the antennae, and the mouthparts are also chemosensory (Ache, 1982; Derby and Atema, 1982c). Little is known about the specificity and sensitivity of chemoreceptors in these latter append-

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ages. Medial antennular filaments differ structurally from lateral filaments: they are shorter, more slender and, most significantly, they do not flick and lack aesthetasc hairs. The latter two features are thought to be important in orientation to chemical gradients (Reeder and Ache, 1980; Devine and Atema, 1982). Nonetheless, in the spiny lobster (*Panulirus argus*) medial filaments contain taurine receptors of equivalent specificity (Fuzessery *et al.*, 1978) and sensitivity (Fuzessery, 1978; Thompson and Ache, 1980) to those found in lateral filaments. Fuzessery (1978) reported that medial filament taurine cells fired more tonically than did lateral filament cells, and suggested that the two filaments may perform different behavioral functions. Thompson and Ache (1980), however, found no difference in firing pattern between medial and lateral filament cells. A resolution of these conflicting observations in *P. argus* has yet to be provided. For other crustacean species, lack of data precludes a comparison of receptor properties of lateral and medial antennular cells.

A complete understanding of how any species uses chemoreception to adapt behaviorally to its environment will require knowledge of the response spectrum and receptor properties (response pattern, tuning breadth, mixture interactions, adaptation and disadaptation rates) of all chemosensory appendages. This study contributes to our understanding of chemoreception in an important model species, the American lobster (*Homarus americanus*), by describing the responses of single chemoreceptor cells from the medial antennular filament to amino acids and related compounds.

Materials and Methods

Medial antennular filaments were excised and mounted in a two-compartment recording chamber. The distal portion of the filament, bearing chemoreceptive sensilla, lay in a cylindrical compartment (inside diameter 3 mm), and was continuously flushed by artificial seawater (ASW) at a rate of 20 ml/min. The lumen of the filament was perfused with oxygenated *Homarus* ringer (Govind and Lang, 1981) through a cannula inserted into the cut distal tip of the filament. Stimuli were injected into the ASW flow above, and perpendicular to, the antennular filament. To estimate the temporal dilution profile of a 50 μl dose of stimulus, we measured the change in conductance of flowing, deionized water (20 ml/min) after injecting 50 μl of a 1 *M* salt solution. Following injection, the stimulus contacted the antennule in approximately .3 s, and peaked within .5 s. At peak the stimulus was diluted 15 times relative to the injected concentration, and was washed out within 1.5 s.

The cut proximal end was immersed in a ringer bath, which was separated from the distal portion of the filament by a sylgard plug. The proximal six annuli were removed to expose the antennular nerve. We used a suction electrode to record extracellular activity from small nerve bundles. Chemoreceptor responses were amplified and displayed by conventional electrophysiological equipment, and stored on magnetic tape.

In the first experiment, we identified chemoreceptive cells by searching for axons that altered their spike activity in response to 50 μl doses of a mixture of 15 compounds (Fig. 1), all at an injected concentration of 10^{-4} *M* (total mixture molarity 1.5×10^{-3}). With the exception of two amino acids (histidine and asparagine), the test stimuli are those used in previous studies of *Homarus* chemoreceptors (Johnson and Atema, 1983; Johnson *et al.*, 1984) and were chosen to allow a direct comparison to the results of these studies. The 15 compounds were then tested singly in doses of 50 μl at 10^{-4} *M* to determine the response spectra for individual cells. Injected concentrations were diluted to approximately 7×10^{-6} *M* in the test chamber. Concentrations reported hereafter account for this dilution. Test compounds, including injections of 50 μl ASW, were presented in varied order with an interval of 60 s between each stimulus application. The mixture was presented at the beginning and end of each test series. We rejected a data series if the final mixture or best single compound presentation elicited a response less than 40% of the initial response (Johnson and Atema, 1983). Chemicals were dissolved in ASW and frozen in 20 ml vials until the day of use. All solutions were presented at room temperature (23 ± 3°C) and at a pH of 7.4 (±.2).

Dose-response relations were determined for cells responding best to hydroxyproline (Hyp), taurine (Tau), and arginine (Arg). Chemicals were tested in an ascending concentration series from 7×10^{-10} to 7×10^{-4}. Because some cells showed marked adaptation to high concentrations of chemicals, we allowed a longer interval of 2 min between stimulus presentations for all dose-response studies.

In the second experiment, we identified arginine-sensitive cells with a search stimulus of 7×10^{-6} *M* arginine. Using the protocol described above, we then tested these cells with each of the arginine analogs listed in Table I.

Response intensity was measured by counting the number of spikes which occurred following stimulus injection. Most responses (75%) were over within 5 s, and the remainder were over within 10 s. A response was considered over when two or more seconds passed without spike activity; occasional spikes outlying this interval were disregarded. Twelve cells were spontaneously active. For these cells, spike counts were corrected for each stimulus presentation by subtracting the number of spikes occurring in a prestimulus interval from the number occurring in a poststimulus interval of equal dura-

Table I

Compounds tested in Experiment 2

Compounds	Structural Formulas
L-Arginine	$COOH(NH_2)CH(CH_2)_3NHC(NH)NH_2$
L-Arginine HCl	$HCl\ COOH(NH_2)CH(CH_2)_3NHC(NH)NH_2$
L-Homoarginine	$COOH(NH_2)CH(CH_2)_4NHC(NH)NH_2$
ω-Nitro-L-arginine	$COOH(NH_2)CH(CH_2)_3NHC(NH)NHNO_2$
L-Arginine methyl ester	$COOCH_3(NH_2)CH(CH_2)_3NHC(NH)NH_2$
Argininic acid	$COOH(OH)CH(CH_2)_3NHC(NH)NH_2$
L-Citrulline	$COOH(NH_2)CH(CH_2)_3NHCONH_2$
L-Ornithine	$COOH(NH_2)CH(CH_2)_3NH_2$
L-Lysine	$COOH(NH_2)CH(CH_2)_4NH_2$
L-Leucine	$COOH(NH_2)CHCH_2CH(CH_3)_2$
L-Isoleucine	$COOH(NH_2)CHCHCH_3(CH_2)CH_3$
L-Norleucine	$COOH(NH_2)CH(CH_2)_3CH_3$
L-Valine	$COOH(NH_2)CHCH(CH_3)_2$
L-Norvaline	$COOH(NH_2)CH(CH_2)_2CH_3$

tion. We identified spikes from single chemoreceptors by analysis of recordings containing only one active cell or, in multiunit recordings, by discriminating cells on the basis of amplitude and waveform. Three cells that did not respond to the mixture (cells 8, 45, and 47; Fig. 2) were identified during the analysis of multiunit recordings.

Results

Experiment 1: general survey

Our analysis of the first experiment used 53 cells, all tested on the complete series of compounds and all clearly resolvable as single units. Best stimuli for the medial receptors were Hyp, Tau, and Arg. Hyp elicited responses from more cells than did any other single compound (Fig. 1A). However, Arg and Tau elicited a greater number of spikes across all cells than did Hyp (Fig. 1B). Three main cell populations were present: Hyp best (14 cells), Tau best (13 cells), and Arg best (11 cells). Also present were cells most responsive to ammonium chloride (NH_4; 4 cells), betaine (Bet; 4 cells), leucine (Leu; 1 cell), lysine (Lys; 1 cell), glutamine (Gln; 1 cell), proline (Pro; 1 cell), glycine (Gly; 1 cell), glutamate (Glu; 1 cell), and the mixture (1 cell).

Tuning properties to single compounds were highly variable, with some cells responsive to only one stimulus and others responsive to several or all stimuli (Fig. 2). Among the Hyp best cells, four (cells 1, 2, 3, and 4) responded only to Hyp. Most of the other cells gave weak responses to one or a few compounds in addition to Hyp, and one relatively broadly tuned cell responded to a total of eleven compounds. Among Tau best cells, four responded only to Tau (cells 15, 16, 17, and 18); five other cells (cells 19, 20, 21, 22, and 23) responded to five or fewer compounds. The remaining four cells (cells 24, 25, 26, and 27) were broadly tuned, responding to eight or more compounds. For Hyp best cells, some compounds were more often stimulatory than were others. For example, asparagine (Asn) elicited spikes from eight Hyp best cells, whereas alanine (Ala) was always nonstimulatory to these cells. However, no compound was consistently the second or third best stimulus for Hyp best cells. Likewise, for Tau best cells there were no consistent next best stimuli.

Arg best cells differed from Hyp best and Tau best cells in at least two major ways. First, Arg best cells generally responded with more spikes (mean number of spikes/response, 136; range, 7–246) than did Hyp best cells (mean number of spikes/response, 27; range, 8–55) or Tau best cells (mean number of spikes/response, 75; range 19–144). Second, Arg best cells were generally broadly tuned and had a consistent second best (Leu) and third best (Lys) stimulus. Some Arg best cells also responded to other compounds, particularly Gln, Bet, Pro, and Ala (Fig. 2); however, the order of effectiveness of these less stimulatory compounds was inconsistent. Although Arg, Leu, and Lys were always the first, second, and third best stimuli, respectively, there was much quantitative variability among cells in how stimulatory Leu and Lys were compared to Arg and to each other. Relative to the response to Arg (100%), the response to Leu ranged from 18% to 94%; the response to Lys ranged from 7% to 57%. The response to Lys, relative to Leu (100%), ranged from 14% to 80%. Because adaptation was common, some of this variability is probably ascribable to the order in which stimuli were presented.

Mixture suppression occurred in most cells (Fig. 3). Responses of Hyp, Tau, and Arg best cells to the mixture were 25%, 38%, and 50%, respectively, relative to responses of these cells to their best compound alone (considered 100%). Responses of NH_4 (32%) and Bet (25%) best cells to the mixture were likewise less than responses to the best compounds alone. Differences were significant for all cell types (Wilcoxon matched-pairs signed-ranks test, $P < .01$).

Dose-response functions for six Hyp and five Tau best cells are shown in Figure 4. One cell responded to Hyp at 7×10^{-10} *M* (response = 6 spikes), but thresholds of other cells were at least an order of magnitude higher. For Tau best cells, one cell responded to Tau at 7×10^{-10} *M* (response = 4 spikes), and most others responded at 7×10^{-9} *M*. All Hyp and Tau best cells had a response range which covered at least three log units.

Experiment 2: arginine cells

We tested the complete series of compounds listed in Table I on 12 cells. Eight cells appeared to be of the same

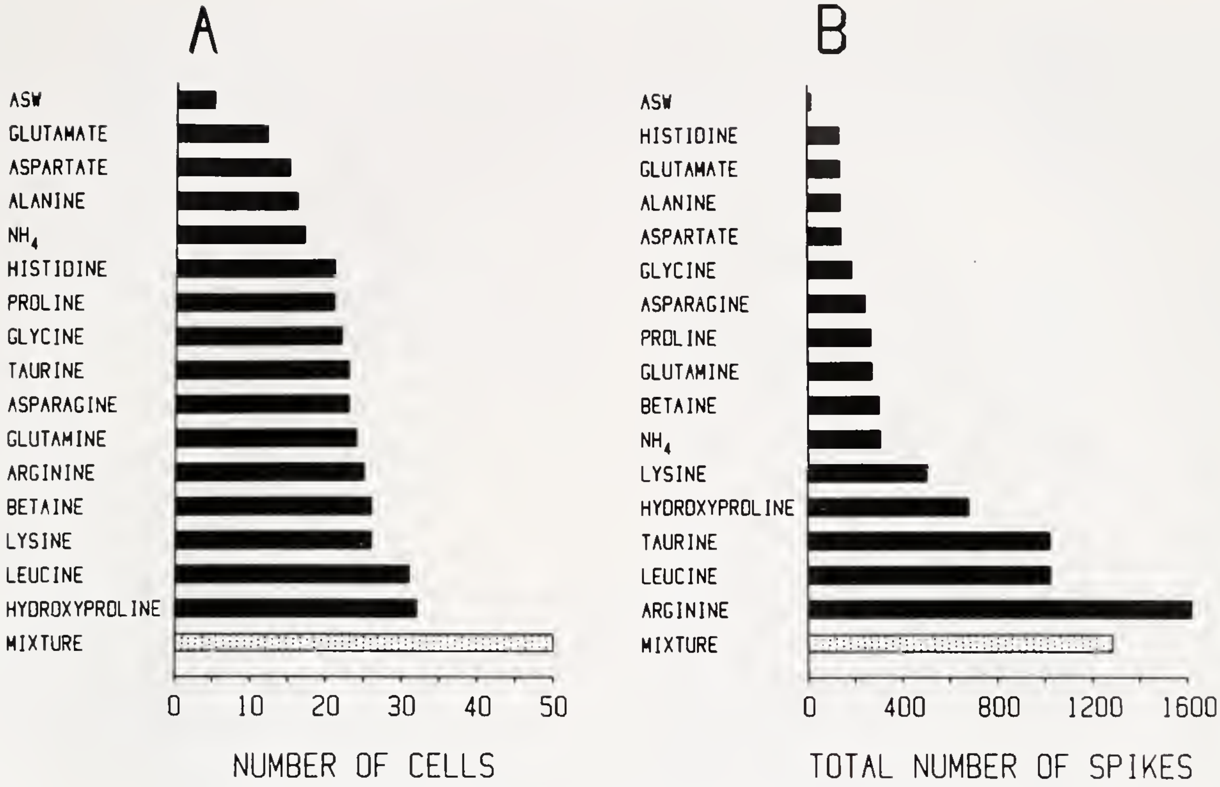

Figure 1. Stimulatory effectiveness of single test compounds, the mixture and ASW on medial antennular filament chemoreceptors from *H. americanus.* All amino acids tested were L isomers. A. Number of cells that responded with one or more spikes to each compound. B. Total number of spikes elicited by each compound (all spikes of all cells).

type identified previously (cells 1–8). These cells generally responded strongly to their best stimulus (mean number of spikes/response, 138; range, 15–283) and were broadly tuned, responding to all or most of the stimuli (Figs. 5, 6). Although these cells were not identical in their responses, a general order of stimulus effectiveness was discernable. Arg and Arg-HCl were always the most stimulatory compounds. Arginine methyl ester, Leu, and isoleucine were typically the third, fourth, and fifth best stimuli, respectively. Other consistently stimulatory compounds were valine, homoarginine, norleucine, norvaline, and Lys. The remaining compounds—citrulline, nitroarginine, ornithine, and argininic acid—elicited weak responses from some cells.

The four additional cells identified by the arginine search stimulus differed qualitatively from the cells described above (Fig. 6). The best stimuli for these cells were argininic acid (cells 9 and 10), Lys (cell 12), and Leu and homoarginine (cell 11). The order of effectiveness of other stimulatory compounds was different in each cell. The response elicited by the best stimulus was much lower in these cells (mean number of spikes/response, 25; range, 5–52) than in the Arg best cells described above.

Discussion

Our results provide the first description of the response spectra of single medial antennular chemoreceptor cells from *Homarus americanus.* These results contribute to a growing literature on how single chemoreceptors respond to amino acids in organisms from bacteria to mammals. More specifically, test stimuli used in the present study included many compounds previously tested on *H. americanus* and *P. argus* lateral antennules and legs (Shepheard, 1974; Fuzessery *et al.,* 1978; Johnson and Ache, 1978; Derby and Atema, 1982a, b; Johnson and Atema, 1983; Johnson *et al.,* 1984), and allow a comparison of response properties of different chemosensory organs. The following properties are briefly discussed below: response spectra of different organs, response magnitude, response duration, tuning breadth, and mixture suppression.

A major similarity in the response spectra of medial and lateral antennular filaments were the prominent populations of Hyp and Tau best cells found in both organs (Shepheard, 1974; Fuzessery *et al.,* 1978; Johnson and Atema, 1983). Like the lateral filaments of *H. americanus* (Johnson and Atema, 1983), the medial filaments

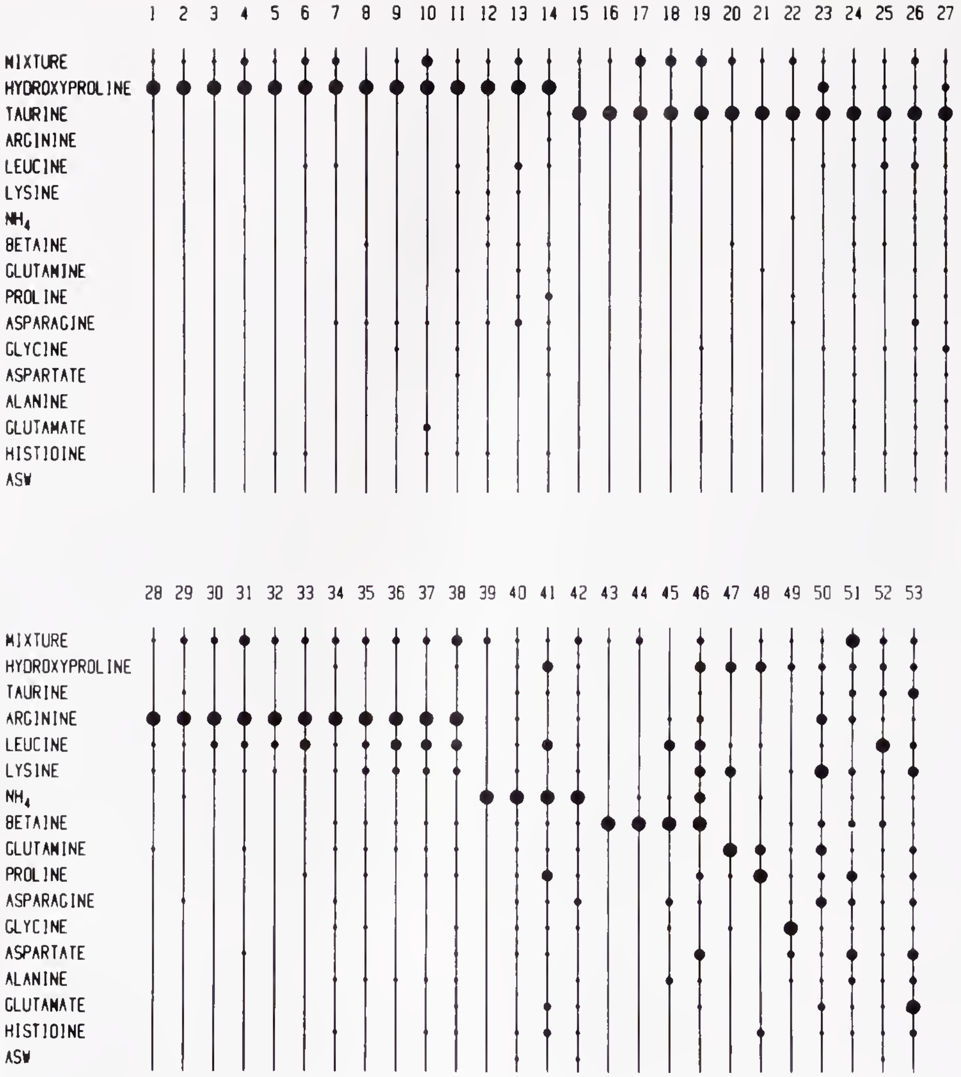

Figure 2. Tuning properties of 53 chemoreceptors from medial antennular filaments. Each cell's responses are normalized to the maximum response. A continuous line indicates no response, the smallest dots indicate less than 30% of the maximum response, second largest dots indicate between 30% and less than 70%, third largest dots indicate between 70% and less than 100%, and the largest dots indicate 100%.

also possessed a few cells most responsive to other compounds (*e.g.*, NH_4, Bet, Pro). Contrary to previous results (Shepheard, 1974), we found that medial filament receptors were not highly responsive to Glu. Medial and lateral filaments also differed in that the former, but not the latter, possessed a significant population of Arg best cells. For leg receptors, best stimuli were Glu, NH_4, Bet and Hyp (Derby and Atema, 1982a, b; Johnson *et al.*, 1984). Smaller populations of cells most responsive to other compounds (*e.g.*, Lys, Tau, Leu: Johnson *et al.*, 1984; Gln, Arg: Derby and Atema, 1982b) were also present. This brief survey demonstrates that, while responses of lobster antennules and legs were not identical, considerable overlap in qualitative sensitivity does appear to exist in the lobster's chemoreceptor organs.

Hyp and Tau best cells from different appendages were similar in the relative magnitude of their responses to their best compound. Hyp best cells in both branches of the antennules typically responded to Hyp with relatively few spikes (mean number of spikes/response to a 1-second pulse of 3×10^{-6} *M* Hyp = 27 for medial antennular receptors and 30 for lateral antennular receptors, Johnson *et al.*, 1985). Responses of Tau best cells were generally stronger (mean number of spikes/response to a 1-second pulse of 3×10^{-6} *M* Tau = 75 for medial antennular receptors and 89 for lateral antennular receptors). The low responses of Hyp best cells suggest that the best stimulus for these receptors may be a compound as yet unidentified. Alternatively, Hyp cells may represent a distinct population of cells that occur in all sensory or-

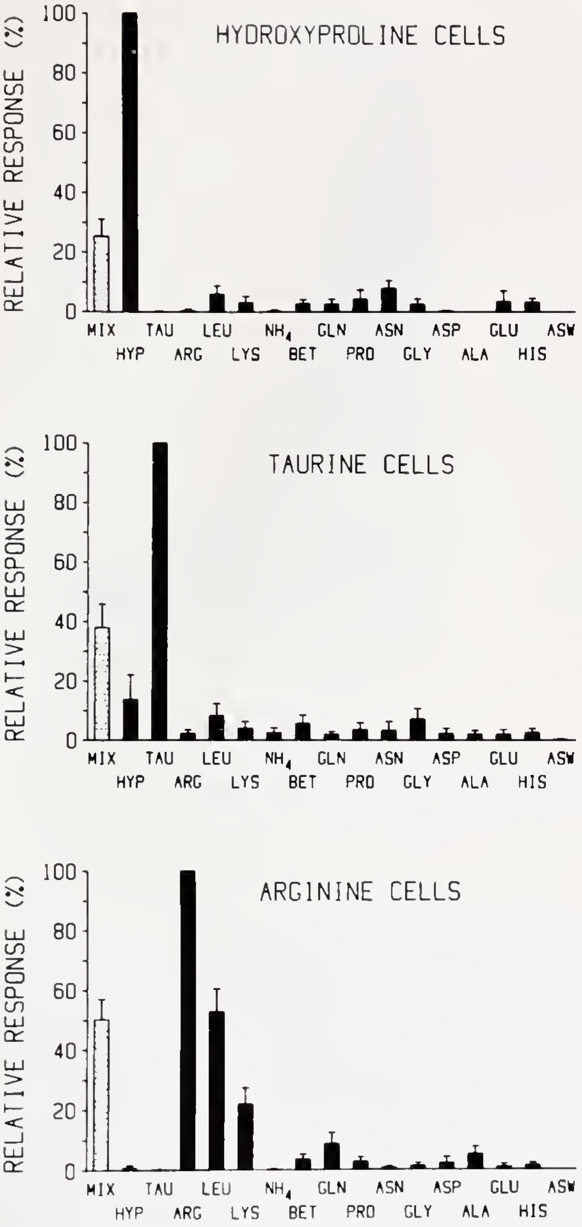

Figure 3. Responses of the three main chemoreceptor cell populations found in the medial antennular filaments of *Homarus americanus*. Bars indicate mean response + SEM of all cells in each population, normalized to the response to the best stimulus (=100%).

gans and which, for reasons perhaps unrelated to stimulus-receptor binding affinities, have a relatively low firing rate (Johnson *et al.*, 1984, 1988).

We found no difference in discharge pattern between medial and lateral filament receptors. Medial antennular receptors typically responded to increasing stimulus concentration with a gradual increase in spike number and response duration (Fig. 4). A similar discharge pattern was observed in *H. americanus* lateral receptors (Johnson *et al.*, 1985). Likewise, Thompson and Ache (1980) found no difference in response duration between medial and lateral filament receptors in *P. argus*. These results contrast with those of Fuzessery (1978) who reported that, for taurine sensitive cells in *P. argus*, responses of medial receptors were tonic, whereas responses of lateral receptors were phasic.

Despite similarities in response spectrum, and in the response magnitude, pattern, and sensitivity of individual cells, the medial and lateral antennular filaments are not behaviorally equivalent. Ablation experiments demonstrated that both *P. argus* (Reeder and Ache, 1980) and *H. americanus* (Devine and Atema, 1982) rely on the lateral filaments for orientation to concentration gradients of food related chemicals. These experiments found that the medial filaments were unnecessary and insufficient for this purpose. At present the function of the medial filaments is unclear, though the results of the present study suggest that the apparent lack of behavioral reliance on medial filaments is not due to a lack of competent receptors. If these structures are in fact behaviorally important as chemoreceptive organs, their importance may be due to the presence of unique receptors not used for orientation within laboratory tanks, and as yet unidentified in physiological tests. Also, the experiments cited above tested animals within two days following lateral antennule ablation. Conceivably, given a longer recovery time, medial filaments may develop a capacity to mediate behaviors such as orientation and thereby functionally replace, at least in part, lost lateral receptors. Such enhancement of chemoreceptor function occurred in crabs in which the sensitivity and behavioral impor-

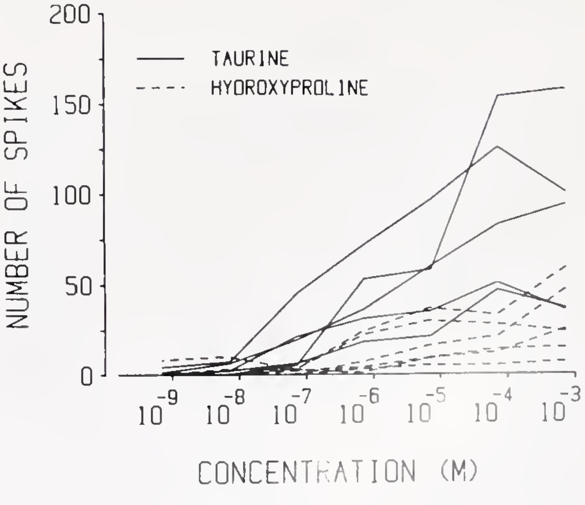

Figure 4. Dose-response functions for six Hyp and five Tau best cells from medial antennular filaments of *Homarus americanus*. Stimuli were tested in an ascending concentration series, with 2 min intervals between stimulus presentations

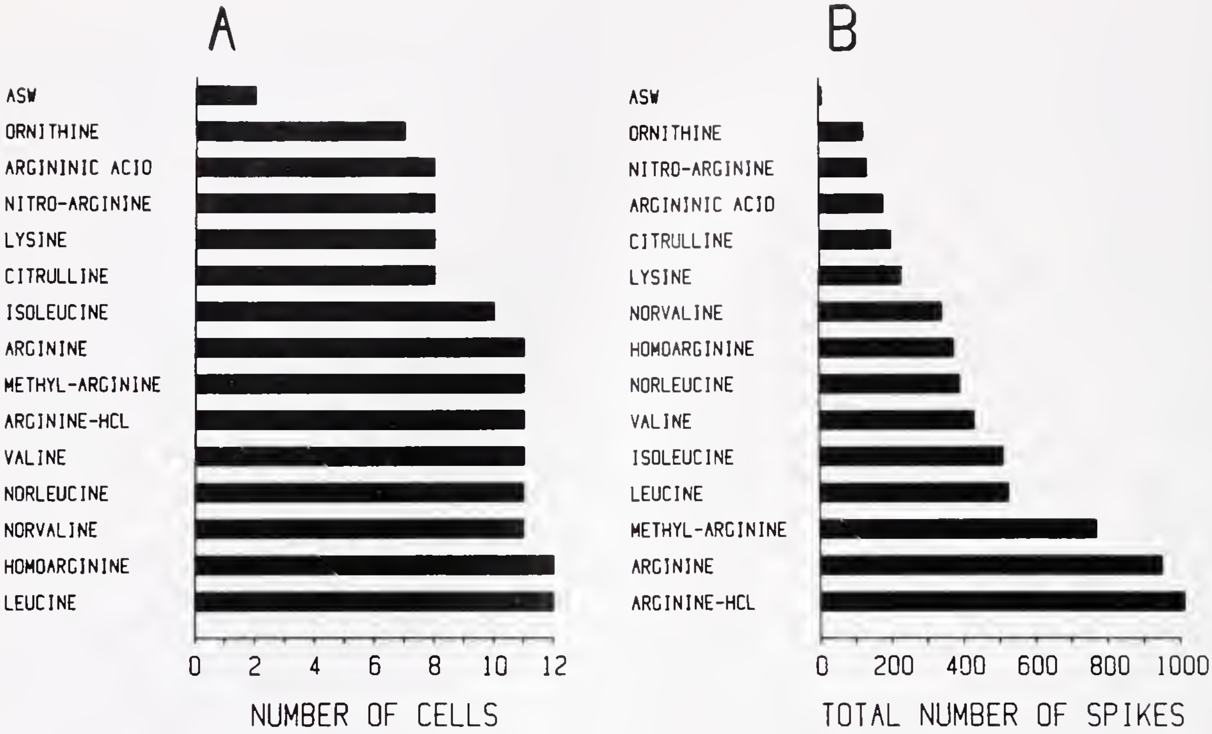

Figure 5. Stimulatory effectiveness of single test compounds and ASW on arginine-sensitive cells in medial antennular filaments from *Homarus americanus.* A. Number of cells which responded with one or more spikes to each compound. B. Total number of spikes elicited by each compound (all spikes of all cells).

tance of dactyl chemoreceptors became increased 7 to 10 days after antennule ablation (Hazlett, 1971).

Narrowly tuned cells are a predominant feature of *H. americanus* lateral antennules (Johnson and Atema 1983) and legs (Derby and Atema 1982b; Johnson *et al.,* 1984). Similarly, many medial filament cells responded strongly to only one compound. Ten cells (four Hyp, four Tau, one Bet, and one NH_4) responded exclusively to one compound (Fig. 2). An additional 14 cells responded only weakly to other compounds (response to a second best compound was less than 30% of the cell's maximal response; Fig. 2). A comparison of H-metric values calculated from the present data and from lateral antennular filaments (data from Johnson and Atema, 1983), indicates that Hyp and Tau best cells are tuned similarly in the two filaments (Table II). The mean H-metric value for Hyp best cells in leg receptors, however, is higher than the values for antennular receptors, suggesting that as a population the former are more broadly tuned than the latter.

Arg best cells had a relatively broad response spectrum and, in contrast to other *H. americanus* chemoreceptor cells, displayed a consistent order of responsiveness to stimulatory compounds. In our initial survey, Leu and Lys were always the second and third best stimuli, respectively. In our second experiment, Leu was less effective than arginine methyl ester, and Lys was less effective than arginine methyl ester, Leu, isoleucine, valine, norleucine, homoarginine, and norvaline. However, despite differences in test compounds, the ratio of responses of Arg, Leu, and Lys were similar in the two experiments. In experiment 1, overall responses to Leu and Lys were 49% and 21%, respectively, relative to the response to Arg (100%); in experiment 2, responses to Leu and Lys were 54% and 22%, respectively, relative to the response to Arg (100%).

Arg best cells from legs were also sensitive to Lys, with Lys being 100 to 1000 times less stimulatory than Arg (Derby and Atema, 1982b). Our dose response data for Arg, Leu, and Lys indicated that medial filament Arg best cells were likewise approximately 100 to 1000 times less sensitive to Lys than to Arg (n = 4; data not shown). Further comparison of Arg cell types must await data on responses of leg receptors to Leu and other compounds stimulatory to medial filament cells. Tau best cells from *P. argus* antennules also had a consistent order of responsiveness to certain Tau analogs (Fuzessery *et al.,* 1978). However, unlike Arg best cells which responded to an array of structurally diverse compounds, Tau cells from *P. argus* responded only to a few compounds closely related to Tau.

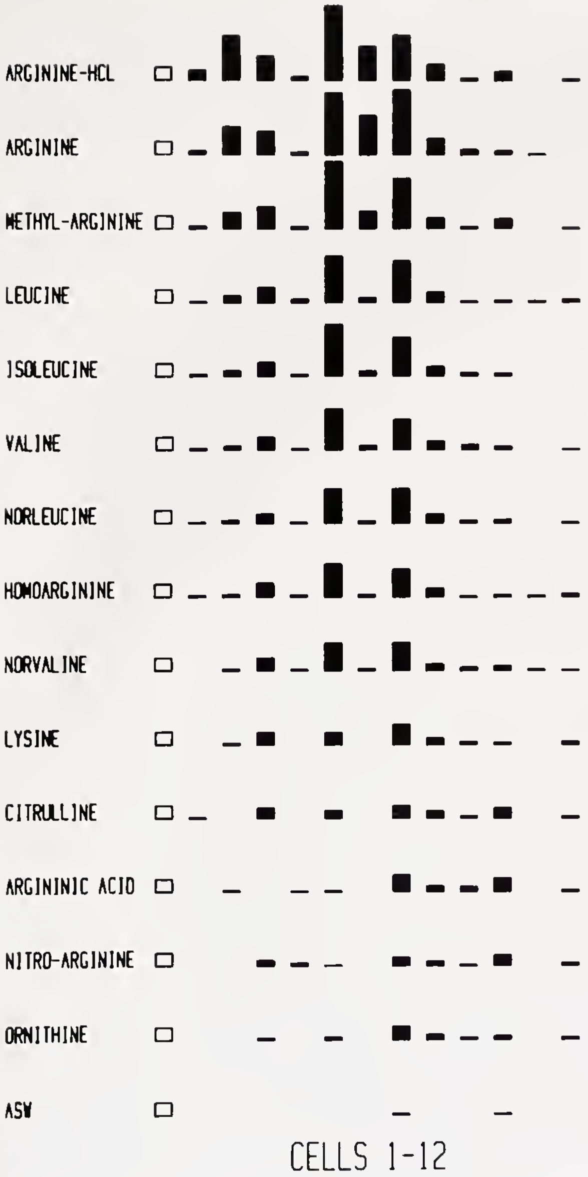

Figure 6. Responses of 12 arginine sensitive chemoreceptors from medial antennule filaments of *Homarus americanus* to 14 single compounds and ASW. Bars indicate the number of spikes each cell gave in response to each compound. Open calibration bars at far left indicate response of 50 spikes.

The functional significance of the distinctively tuned Arg best cells is unknown. Amino acid receptors of aquatic animals are commonly assumed to function as detectors of food related compounds. Narrow tuning may enhance the ability of receptors to perceive important stimuli by reducing interference from other possibly cross-adapting compounds (Fuzessery *et al.*, 1978; Johnson *et al.*, 1984). It is not clear, however, why this explanation applies only to certain amino acids, and only to certain species. In crayfish leg receptors, for example, all amino acid sensitive cells were broadly tuned, with the order of stimulus efficacy consistent from cell to cell (Bauer *et al.*, 1981; Hatt, 1984). Chemoreception studies necessarily test only a minute fraction of potentially stimulatory compounds. Conceivably the striking difference in tuning breadth between Arg best cells and other *H. americanus* cells is an artifact resulting from the selection of test compounds. Thus, perhaps Glu best cells possess second, third, *etc.* best stimuli, but the appropriate compounds were not included in tests which defined the response spectrum of these cells.

Mixture interactions, both synergistic and suppressive, occur in the chemoreceptor systems of invertebrates and vertebrates (Shiraishi and Kuwabara, 1970; Bartoshuk, 1977; Dethier, 1977; Hyman and Frank, 1980a, b; Cagan, 1981; Gillan, 1982, 1983). In the lobster olfactory pathway mixture suppression occurs in peripheral receptors (Johnson and Atema, 1983; Gleeson and Ache, 1985; Johnson *et al.*, 1985) and central interneurons (Derby and Ache, 1984a, b). Examples of peripheral suppression are Hyp and Tau best cells from lateral antennular filaments which responded better to their best compound alone than to this compound within a mixture (responses were 58% for Hyp and 69% for Tau within the mixture, relative to 100% for responses to the best compound alone; Johnson and Atema, 1985). Mixture suppression in medial filament cells was even more pronounced for Hyp and Tau best cells (responses to these compounds within the mixture were 25% and 38%, respectively; response to compounds alone = 100%). Arg best cells, however, showed less pronounced suppression (50% = Arg alone; 100% = mixture). Possibly the latter phenomenon is attributable to the presence within the mixture of at least two additional compounds (Leu and Lys) which reliably, and often substantially, stimulated Arg best cells. Thus, during mixture presentations Leu

Table II

Response breadth of hydroxyproline and taurine best cell populations in the medial antennular filament, lateral antennular filament and the legs of Homarus americanus *measured by the H-metric**

Cell populations	Mean	Median	Range	N
Hyp (medial)	.1906	.1674	0–5744	14
Hyp (lateral)	.2190	0	0–.8717	17
Hyp (leg)	.4376	.4307	0–.6841	9
Tau (medial)	.2682	.1021	0–.8746	13
Tau (lateral)	.2399	.1328	0–.8822	7

N is the number of cells.

* $H = -K \sum_{i=1}^{n} P_i \log_{10} P_i$; where H = entropy measure of response breadth, K = scaling constant (−0.8977, for 13 compounds; sucrose, ethanol, Asn and His were eliminated from the analysis because they were not used in all three studies), P_i = the proportional response of each of the 13 test compounds (Smith and Travers, 1979).

and Lys could occupy receptor sites and cause moderate cell stimulation while excluding a proportion of the suppressive compounds contained in the mixture.

Acknowledgments

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Chemoautotrophic Symbiosis in a Hydrothermal Vent Gastropod

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Abstract. An undescribed gastropod species collected from recently discovered deep-sea hydrothermal vents in the western Pacific contains endosymbiotic bacteria within specialized gill cells. The snails inhabit rocky vent openings where they are exposed directly to warm (2–25°C) sulfide-rich (750 μM) water emitted from the vents. The gills of this snail contain elemental sulfur and high activities of enzymes catalyzing sulfide metabolism (sulfide oxidase, ATP-sulfurylase, APS-reductase, rhodanese) and autotrophic CO_2 fixation (ribulose bisphosphate carboxylase) indicating that the bacteria function as sulfur oxidizing chemoautotrophic endosymbionts—a symbiosis described previously only in vestimentiferan and pogonophoran tubeworms, oligocheate worms, and bivalve molluscs. This represents the first documentation of chemoautotrophic potential among the numerous gastropod species found inhabiting the interface of reducing and oxidizing environments.

Introduction

Molluscs of the class Gastropoda obtain nutrition primarily as grazers, predators, or deposit feeders. In a recent expedition to the spreading center in the Mariana Back-Arc Basin, (western North Pacific 18° 11′ N, 144° 43′ W), dense communities of animals were discovered surrounding deep-sea hydrothermal vents. Unlike previously described hydrothermal vent communities in the eastern Pacific, where large vestimentiferan tube worms and bivalve molluscs are the most conspicuous organisms (Hessler and Smithey, 1983), at the Mariana vents dense concentrations of an undescribed mesogastropod predominate at rocky vent openings (Fig. 1). Measurements showed that these snails were exposed directly to emerging vent water with temperatures up to 25°C and concentrations of hydrogen sulfide of 750 μM (K. Johnson, University of California, Santa Barbara, pers. comm.).

Because many bivalve molluscs that are exposed directly to sulfide-rich water house chemoautotrophic sulfide-oxidizing bacteria as symbionts within modified gills (Felbeck *et al.*, 1981; Reid and Brand, 1986; Southward, 1986), we suspected that the snails may have evolved a similar strategy. We also considered the possibility that the snails housed methanotrophic bacteria as symbionts, a more recently discovered relationship in mytilids inhabiting vent-type communities (Childress *et al.*, 1986; Cavanaugh *et al.*, 1987). Unlike bivalves, however, which can filter large volumes of water and thereby readily provide their endosymbionts with the dissolved nutrients required for chemoautotrophy, the gastropods, with their tightly coiled shells, seemed at first unlikely candidates to house similar endosymbionts. Yet we now present microscopic and enzymatic evidence for a sulfide-based chemoautotrophic symbiosis in the class Gastropoda.

Materials and Methods

Specimens for this study were collected at a depth of approximately 3650 m at the "Snail Pit" site by the sub-

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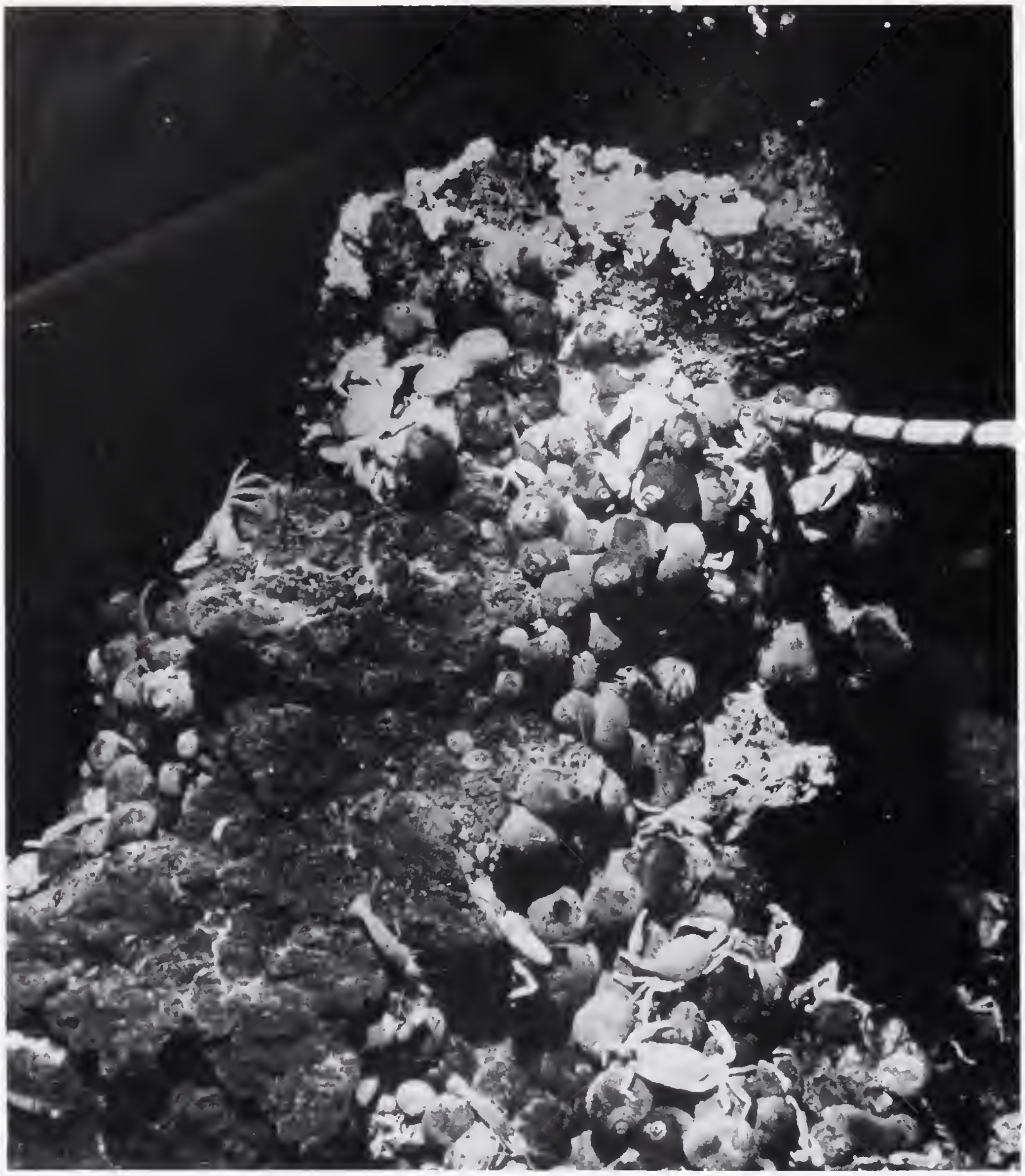

Figure 1. Mariana gastropods encrusting an active hydrothermal chimney at 3632 m depth in the Mariana Back-Arc Basin. Large snails are approximately 5 cm in diameter. The temperature probe at top right measured temperature anomalies of 2–20°C where the snails live. Limpets, caridean shrimp, and brachyuran crabs are common associates of the snail.

mersible *Alvin* and transported in an insulated container to the surface where individual snails were either dissected and fixed for transmission electron microscopy (TEM) or frozen immediately in liquid nitrogen for enzyme assays. A morphological and taxonomic description of the snail is being prepared by S. Ohta and T. Okutani, Tokyo University of Fisheries, Tokyo 108, Japan.

Microscopy

Gill squash preparations for light microscopy were prepared by thawing approximately 50 mg of frozen tissue at room temperature in a sterile seawater solution containing 0.1 μg/ml of the specific DNA stain 4,6-diamidino-2-phenylindole (DAPI). The tissue was pressed under a glass cover slip and viewed under epifluorescence microscopy according to the methods of Porter and Feig (1980).

Tissues for transmission electron microscopy (TEM) examination were fixed freshly with 2% glutaraldehyde/0.5% formaldehyde in 75 m*M* cacodylate buffer (pH 7.4, 7.5% w/v sucrose). After rinsing with buffer, tissues were post-fixed in 1% osmium tetroxide, dehydrated in a graded ethanol series followed by propylene oxide, then embedded in Epon 812. Thin sections were cut with a diamond knife, stained with lead citrate and uranyl acetate, and examined and photographed with a JEOL-100CX electron microscope.

Enzyme and elemental sulfur measurements

For enzyme assays and measurements of percent elemental sulfur, tissues frozen in liquid nitrogen for 2 to 5 weeks were transferred to a −80°C freezer where they were stored for approximately 3 weeks prior to analyses. We determined the metabolic potential of the bacterial endosymbionts by assaying for enzymes responsible for sulfur oxidation and subsequent ATP generation (ATP-sulfurylase [E.C. 2.7.7.4], APS-reductase [E.C. 1.8.99.2], rhodanese [E.C. 2.8.1.1], and for the ATP dependent fixation of carbon dioxide via the Calvin-Benson cycle (ribulose bisphosphate carboxylase (RuBPCase) [E.C. 4.1.1.39]). ATP-sulfurylase was measured with the method of Felbeck (1981), APS-reductase according to Peck *et al.* (1965), rhodanese according to Smith and Lascelles (1966), and RuBPCase according to Wishnick and Lane (1971). Activities of this suite of enzymes have been used in the past as a diagnostic tool to assess the chemoautotrophic potential of invertebrates containing putative sulfur-oxidizing symbionts. The ability of the snails to oxidize and thereby detoxify sulfide was determined by assaying for activity of "sulfide oxidase" with the method of Powell and Somero (1985). As a final determinate of sulfur metabolism, we measured an intermediate product of sulfur oxidation—elemental sulfur—in the gill and foot tissues of the snail using two methods—a spectrophotometric method of Schedel and Trüper (1980) as modified by Vetter (1985) and a gas chromatographic method of Richard *et al.* (1977) as modified by Fisher *et al.* (1987). We tested for methylotrophic symbionts by assaying for methanol dehydrogenase [E.C. 1.1.99.8] using the spectrophotometric method of Anthony and Zatman (1965).

Gill and foot tissues from five snails were each analyzed separately for RuBPCase, sulfide oxidase, ATP-sulfurylase and the spectrophotometric determination of percent elemental sulfur. Assays for rhodanese, APS-reductase, methanol dehydrogenase, and the gas chromatographic measurement of percent elemental sulfur were performed on tissues from different individuals. For all enzymes, except for methanol dehydrogenase, frozen trophosome tissue from the vestimentiferan tubeworm *Riftia pachyptila* was used as the positive control. Gill tissues of an unidentified mytilid, demonstrated to harbor methanotrophic symbionts (Childress *et al.*, 1986), served as the positive control for methanol dehydrogenase.

Results

Examination of dissected snails revealed that the gills were extremely hypertrophied, lobular in shape, light in color, and comprised approximately 40% of body volume. Viewed under epifluorescence microscopy, DAPI-stained gill-squash preparations contained abundant rod-shaped, membrane-bound bodies approximately 5 μm in length which fluoresced blue. When examined under polarizing light, these putative symbionts contained spherical, refractile inclusions reminiscent of the liquid crystalline sulfur inclusions described in the symbiotic sulfur bacteria of certain bivalves inhabiting sulfide-rich environments (Vetter, 1985). Indeed, the elemental sulfur content in the gills was comparable to that of the vent bivalve *Calyptogena magnifica* and in the trophosome tissue of the vestimentiferan tubeworm *Riftia pachyptila* (Table I).

TEM examination of thin transverse sections taken across gill filaments revealed that the putative symbionts contained no membrane-bound organelles and possessed a non-membrane-bound nuclear region thus supporting the view that they are prokaryotic. The bacteria were packed densely within microvilli-fringed cells (Fig. 2), resembling closely the arrangement of bacteria within the bacteriocyte cells of symbiont-containing bivalve mollusc gills (Reid and Brand, 1986).

We found activities of ATP-sulfurylase, rhodanese, RuBPCase, and sulfide oxidase in gill homogenates of the Mariana vent snail comparable to levels reported for *C. magnifica* and for *R. pachyptila* although no significant activity of APS-reductase was detected with the methods used (Table I). With the exception of the low activity of sulfide oxidase, snail foot tissue and control assays without respective substrate showed no activity for any of these enzymes. Methanol dehydrogenase activity was not detected.

Discussion

The presence of bacteria within specialized gill cells in the Mariana gastropod coupled with the activities of enzymes responsible for fixing carbon dioxide and extracting energy from sulfide represent the first documentation of chemoautotrophy in the class Gastropoda. Comparable morphology and enzyme levels have been described previously only in symbiont-bearing bivalves and tubeworms which have been demonstrated to fix carbon dioxide into metabolites of intermediary metabolism (Felbeck, 1983, 1985). Another vent gastropod, an unde-

Table I

Percent elemental sulfur (dry weight) and enzyme activities in tissues of the Mariana gastropod *and other hydrothermal vent taxa*

	Mariana gastropod (n = 5)		*Calyptogena magnifica* (Bivalvia)	Riftia pachyptila (Vestimentifera)
	Gill	Foot	Gill	Trophosome
Elemental sulfur				
a) spectrophot.	4.4 ± 1.4	N.D.	NA	NA
b) gas chrom.	2.8 ± 1.0§	N.D.	4.4*	13.5*
ATP-sulfurylase	42.6 ± 8.4	N.D.	2.5#	74**
APS-reductase	N.D. (n = 2)	N.D.	NA	23.3**
Rhodanese	25.9 (n = 2)	N.D.	NA	7.6**
RuBPCase	0.2 ± .02	N.D.	0.4**	0.22**
Sulfide oxidase	5.3 ± 1.4	0.4 ± 0.3	6.1 ± 2.1 (n = 22)##	31.7 ± 8.8 (n = 8)##
MeOH dehydrog.	N.D. (n = 2)	NA	N.D.	N.D.

Elemental sulfur measurements were determined on freeze-dried tissues. Enzyme activities are given in international units (μmoles of substrate converted to product per min) per g wet weight ± standard deviation (where applicable). N.D.: not detectable; NA: not available. Detection levels (in units per liter of extract) for "N.D." enzymes are: ATP-sulfurylase (10), APS-reductase (0.01), Rhodanese (5), RuBPCase (50), and MeOH dehydrog. (25).

§ n = 5.

* J.J.C., pers. obs.

** Felbeck *et al.*, 1981.

# H.F., pers. obs.

## Fisher and Childress, 1984.

scribed limpet from the Juan de Fuca Ridge, has been shown to endocytose bacteria that colonize the gill epithelium (deBurgh and Singla, 1984). However, in this case the bacteria are not contained within bacteriocyte cells but appear to be immediately degraded by lysosome-like cells and, therefore, are probably not involved in a symbiotic association with the limpet.

The proportion of the Mariana gastropod's metabolism that is fueled by symbiont-derived carbon is not yet known, for they also possess an apparently functional radula and gut although no individuals examined thus far contained a significant amount of material within their gut. Sacoglossan gastropods of the family Elysiidae also maintain a functional radula and gut yet derive most of their energetic requirements from photosynthetically fixed carbon of functional chloroplasts acquired from ingested algae (Trench, 1975). Further, the tight clustering of the snails around the vents, the greatly enlarged and modified gill, and the high enzyme activities relative to those expected in a strictly heterotrophic organism, in combination suggest that the snails derive nutritional or energetic benefit from the symbiosis. The snails may alternatively rely on their symbionts principally for detoxification of sulfide thus allowing them to graze upon epilithic bacteria closer to the vents. However, gastropods that graze on sulfur bacteria at shallow-water vents (Stein, 1984) do so without benefit of sulfide detoxification by bacterial symbionts.

Although the oxidation of sulfide results directly in ATP synthesis by the bacteria of *Riftia pachyptila* (Powell and Somero, 1986a), and in the mitochondria of the gutless protobranch clam, *Solemya reidi* (Powell and Somero, 1986b), sulfide is also a potent respiratory inhibitor whose entrance, transport, and metabolism—even in vent organisms—must be tightly controlled (Powell and Somero, 1986b; Vetter *et al.*, 1987). The low level of sulfide oxidizing activity in the snail's foot tissue relative to the gill suggests that "sulfide oxidase" activity may be restricted to the superficial layers of the foot as is found in *S. reidi* (Powell and Somero, 1986a) or that the animal tissue may be protected by another defense mechanism, perhaps a circulating sulfide binding protein as in the blood of *R. pachyptila* (Arp and Childress, 1983; Fisher and Childress, 1984). However, equilibrium dialysis of the gastropod blood revealed no such binding activity.

Also indicative of the prokaryotic metabolism of sulfide is the high level of elemental sulfur in the snail's gill. It has been suggested that elemental sulfur stored by bacteria within the gills of symbiont-containing bivalves represents an intermediate product of sulfide oxidation that may serve as an energy storage reserve which can be oxidized to produce energy in the absence of ambient sulfide (Vetter, 1985). Since deep-sea hydrothermal vents can be locally ephemeral features (Grassle, 1986), such an energy reserve might benefit a motile gastropod which

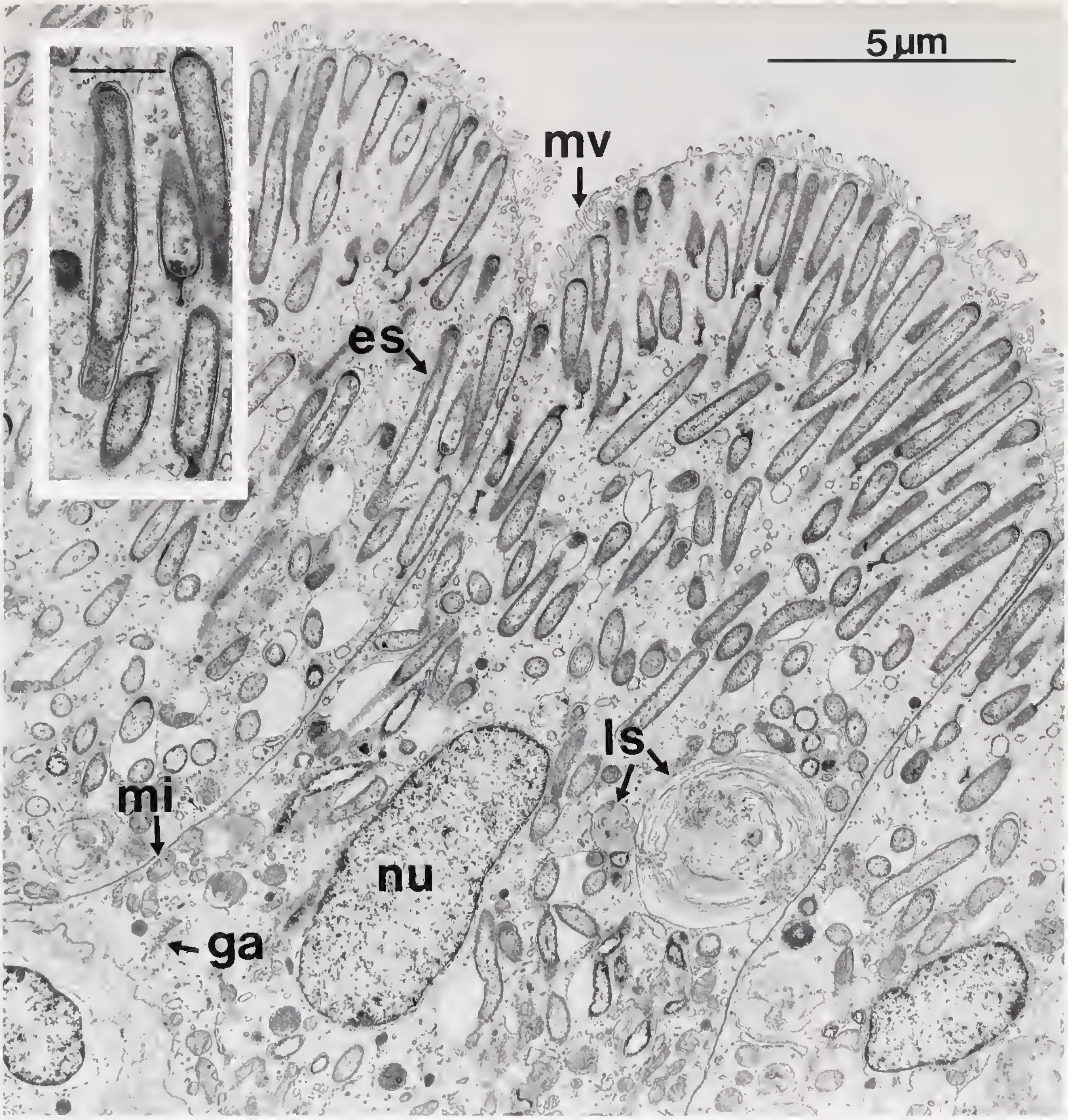

Figure 2. Transmission electron micrograph showing rod-shaped endosymbiotic bacteria in the bacteriocytes of the gill of the Mariana gastropod. Nuclei of the bacteriocytes (nu), lysosomes (ly), mitochondria (mi), and Golgi apparatus (ga) are found in the basal regions of the cells while the slender endosymbionts (es) are typically observed in the outer region of the cells arranged in a radial fashion. The outer surface of the bacteriocyte is covered with microvilli (mv). *Inset.* Enlargement of endosymbionts showing cell membranes (scale bar = 1 µm.).

could find more suitable habitat in the face of diminishing vent flow.

Endosymbioses between sulfur bacteria and bivalve molluscs have been described worldwide from deep-sea hydrothermal vents, sewage outfalls, mangrove swamps, and other environments where there is simultaneous access to reduced sulfur compounds and molecular oxygen (Cavanaugh, 1983; Felbeck, 1983; Schweimanns and Felbeck, 1985). Although gastropods occur in many of these habitats, and large gastropod shells have been described at hydrothermal vents in the Manus Back-Arc basin near New Guinea (Both *et al.*, 1986), none until now have been reported to contain bacterial endosymbionts. From evolutionary and biogeographical standpoints, it is of interest that gastropod endosymbiosis has apparently not attained the wide distribution reported in bivalves and has not been reported outside the western Pacific. Nonetheless, the chemoautotrophic potential of the Mariana gastropod suggests that additional examples of similar symbioses within this and other taxa may yet be found.

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